2006

Stretch-Dependant Tonic Force Maintenance in Rabbit Epigastric Artery

Krystina Michelle Berg
Virginia Commonwealth University

STRETCH-DEPENDANT TONIC FORCE MAINTENANCE IN

RABBIT EPIGASTRIC ARTERY

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

KRYSTINA MICHELLE BERG
B.S., Old Dominion University, 2001

Director: PAUL H. RATZ
PROFESSOR
DEPARTMENT OF BIOCHEMISTRY

Virginia Commonwealth University
Richmond, Virginia
June 2006

Acknowledgement

After four long years of research, life learning and emotional growth, I would like to thank those who have given me wisdom along the way. First of all, I would like to thank my committee members for all of their time, patience and advice. I would like to thank Dr. Ratz for encouraging me to pursue my graduate education and providing me with ample space and equipment to get the job done. I would especially like to thank Dr. Diegelmann, who has served over the past several years not only as a mentor but my own personal guidance counselor when it all got a little rough. I would also like to express great appreciation to not only my friend, co-worker and mentor, but also my personal editor always available for review, mental exercise and even a lunch date, Melissa Evans. She and the other members of the lab Nicki Urban, Tracey Webb and Lindsey Borgsmiller have always made the time of being a 'lab rat' much more enjoyable.

I would like to thank my family for their unconditional support, my mom Debbie, my brothers Troy and Jason Berg and especially my future husband, Brandon Hazelgrove. Every night he has listened to my elation and disappointment during the course of this dissertation and has made my commitment to him that much stronger.

Finally, I would like to dedicate this dissertation to my father, Jon Michael Berg, who passed away quite unexpectedly during the course of this research. His love was always shown not through his words, but his constant pushing to be the best and do the

best. Although he showed tough love, his stubbornness rubbed off on me and without it I might never have been able to finish. Dad I know that you are proud of me.

Jon Michael Berg 1951-2003

Table of Contents

List of Figures

Page

List of Abbreviations

CaD.....Caldesmon
CaM.....Calmodulin
CaMKII.....Calmodulin Kinase II
cAMP.....cyclic AMP
CaP.....Calponin
cGMP.....cyclic GMP
EA.....Epigastric Artery
ERK.....Extracellular Regulated Kinase
FA.....Femoral Artery
ILK.....Integrin-Linked Kinase
IP_3.....nositol 1,4,5-trisphosphate
MAPK.....Mitogen-Activated Protein Kinase
MLC.....Myosin Light Chain
MLC-p.....Phosphorylated Myosin Light Chain
MLCP.....Myosin Light Chain Phosphatase
MT.....Microtubule
MYPT.....Myosin Phosphatase Targeting Subunit
PAK.....p21-Activated Kinase
PKA.....Protein Kinase A
PKC.....Protein Kinase C
PKG.....Protein Kinase G
PP1.....Protein Phosphatase type 1
RhoGAP.....GTPase Activating Protein
RhoGEF.....Guanine nucleotide Exchange Factor
ROK.....RhoA Kinase
RyR.....Ryanodine Receptor
smMLCK.....smooth muscle Myosin Light Chain Kinase
SOC.....Store Operated Channel
SR.....Sarcoplasmic Reticulum
TRP.....Transient Receptor Potential
VOC.....Voltage Operated Channel
VSM.....Vascular Smooth Muscle

Abstract

LENGTH-DEPENDANT TONIC FORCE MAINTENANCE IN

RABBIT EPIGASTRIC ARTERY

By Krystina Michelle Berg, B.S.

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2006

Major Director: Paul H. Ratz
Professor
Department of Biochemistry

The contractile state of vascular smooth muscle (VSM) plays a key role in blood pressure regulation. Abnormal VSM contractility characterizes hypertension and understanding the regulatory mechanisms of VSM may provide some insights to specific treatment of hypertension. Upon muscle stimulation, Ca^{2+}, myosin light chain (MLC) phosphorylation, crossbridge cycling rates and force increase to high levels, but with time, force is maintained while all other parameters of muscle activation fall to low levels. Thus, contraction is divided temporally into early (phasic) and late (tonic) phases, as determined by the underlying regulatory mechanism. Muscles with more

phasic characteristics have a higher peak phase while tonic muscles have both high peak and tonic. However, these muscles have similar contractile increases in Ca^{2+} despite their substantial differences in force production during the tonic phase of contraction. Myosin light chain phosphatase (MLCP) inhibition by RhoA Kinase (ROK) has been shown to contribute to this increased force production without simultaneous increases in calcium in a process known as Ca^{2+} sensitization.

Epigastric artery (EA) has a unique regulatory mechanism controlled by the degree of stretch applied on the artery which produces an increase in tonic force maintenance. Tonic force in EA is blocked equally at all lengths by the ROK inhibitor Y-27632, suggesting ROK is the main mechanism of signal transduction activated. MLC-p is increased during basal compared to unstretched conditions for epigastric but not femoral artery (FA). Pharmacological MT depolymerization due to Colchicine incubation has been shown to release RhoGEF, a RhoA activator, and resulted in increased tonic force and MLC-p which were both inhibited by a ROK inhibitor. Additionally, KCl-stimulation appeared to activate MAPK and ROK pathways, while stretch alone activated a yet undetermined pathway, possibly ILK. KCl-induced contraction in FA activated TRP sensitive calcium channels during both peak and tonic phases. However, stretch in EA does not induce additional calcium influx. Thus, these data support the conclusion that an increase in length activates ROK and other kinases resulting in tonic force maintenance in EA.

Chapter 1: Background

Contractile Apparatus of Vascular Smooth Muscle

Smooth muscle contraction is an essential action to many organ systems in the body including the vascular system, gastrointestinal system, urinary track and the bladder. Smooth muscle must maintain a high level of contraction at low energy consumption in order to maintain certain bodily functions, such as blood pressure maintenance in the case of the vascular system. Muscles which have a high level of energy conservation are defined as having both low shortening velocity and low energy consumption (Dillon et al. 1981; Paul 1990; Wingard et al. 1997; Hai and Murphy 1988). These qualities distinguish smooth muscle from the fast, but energy consuming skeletal muscle. However, not all smooth muscle organ systems contain myosin isotypes with the same level of energy consumption. Somlyo and Somlyo proposed in 1968 that smooth muscle contraction can be divided into a fast, unsustained phasic and a slower, more sustained tonic phases. Additionally, the rate of shortening velocity in smooth muscle can range seven-fold from slowest to fastest smooth muscle isotype, a greater range than between fast and slow skeletal muscle (Malmqvist and Arner 1991).

Contraction initiated by receptor agonists, high K^+ or stretch in myogenic contractions elicit an increase in cytosolic calcium concentration ($[Ca^{2+}]_i$). Increased $[Ca^{2+}]_i$ results in activation of myosin light chain kinase (MLCK) through calmodulin

(CaM) (Fig 1) (Kamm and Stull 1985). CaM is a cofactor required to activate MLCK and binds to four Ca^{2+} ions. Once the regulatory Ca^{2+}/CaM subunit is formed, it can then bind to and activate the catalytic subunit of MLCK to phosphorylate the regulatory subunit of myosin, myosin light chain 20 (MLC_{20}), on the primary phosphorylation sites of Ser-19 and Thr-18 (Fig 1). Phosphorylation of MLC_{20} then activates myosin ATPase activity and increases maximal shortening velocity of actin/myosin crossbridges thus increasing contractile force (reviewed by Walsh 1994) (Fig 2). Removal of $[Ca^{2+}]_i$ results in decreased activity of MLCK and decreased MLC_{20} phosphorylation due to the continued activity of myosin light chain phosphatase (MLCP).

Contraction in smooth muscle occurs by two main mechanisms including pharmacomechanical and electromechanical coupling(Somlyo and Somlyo 1968). Pharmacomechanical coupling of excitation and contraction is a combination of mechanisms which induce contraction through calcium channels independent of membrane potential. Electromechanical coupling refers to calcium influx through voltage dependant channels activated by changes in membrane potential. These two mechanism may be activated simultaneously during agonist induced contractions(Somlyo and Somlyo 1968). However, by using KCl as the contractile agent in the experimental model, we sought to study mechanisms activated through electromechanical coupling of calcium release and muscle contraction.

Smooth muscle myosin II is a hexamer consisting of two light chains and two heavy chains. Alternative splicing allows for four alternate heavy chains: SM1-A, SM2-A, SM1-B, and SM2-B. SM1 differs from SM2 by approximately 43 unique amino

acids in the C-terminal region which is truncated to 9 AA's in the SM2 form(Eddinger and Murphy 1988). The expression of these two isoforms varies during development and hypertrophy (Eddinger and Murphy 1991), but does not affect the interaction with actin (Meer and Eddinger 1997). However, the presence (SMB) or absence (SMA) of a 7 AA insert in the loop-1 region at the 25/50kDa domain junction region of the head produces an increase in maximal shortening velocity due to its proximity to the catalytic site of the myosin heavy chain(Kelly et al. 1993). The insert can be present in either SM1 or SM2 generating the four separate isoform combinations. Myosin of subtype SM1 or SM2 also containing SMB have been shown to induce maximal shortening velocity and faster force production. Additionally, two splice variants of the RLC_{17b} subtype results in decreased ATPase activation (Hasegawa and Morita 1992). Therefore, the combination of LC17a and SMB results in a faster smooth muscle crossbridge cycling rate.

Evidence for Calcium Sensitivity

Increases in $[Ca^{2+}]_i$ lead to increase MLCK activity, phosphorylation of MLC_{20} and subsequent force production (Fig 1)(Kamm and Stull 1985). Although calcium is a cellular regulator of many processes, force production is further modulated by changes in calcium sensitivity. In other words, the force response observed at a given level of calcium may either increase or decrease depending on the signaling pathway activated in addition to calcium signaling. An increase in force production at a given level of calcium is termed calcium sensitization, whereas a decrease in force for a fixed level of

Figure 1. Model of Vascular Smooth Muscle Contraction Force production in vascular smooth muscle occurs through activation of the contractile proteins. Activation of myosin ATPase activity and subsequent interaction with actin and force production is initiated by calcium influx and activation of myosin light chain kinase (MLCK) resulting in phosphorylation of myosin light chain and activation of myosin ATPase. Activation of myosin light chain phosphatase (MLCP) results in dephosphorylation of myosin light chain and thus inhibition of contraction. Calcium sensitization mechanisms inactivate MLCP to cause an imbalance in the kinase to phosphatase activity ratio. Such mechanisms therefore allow for an increase in force for the same level of calcium influx.

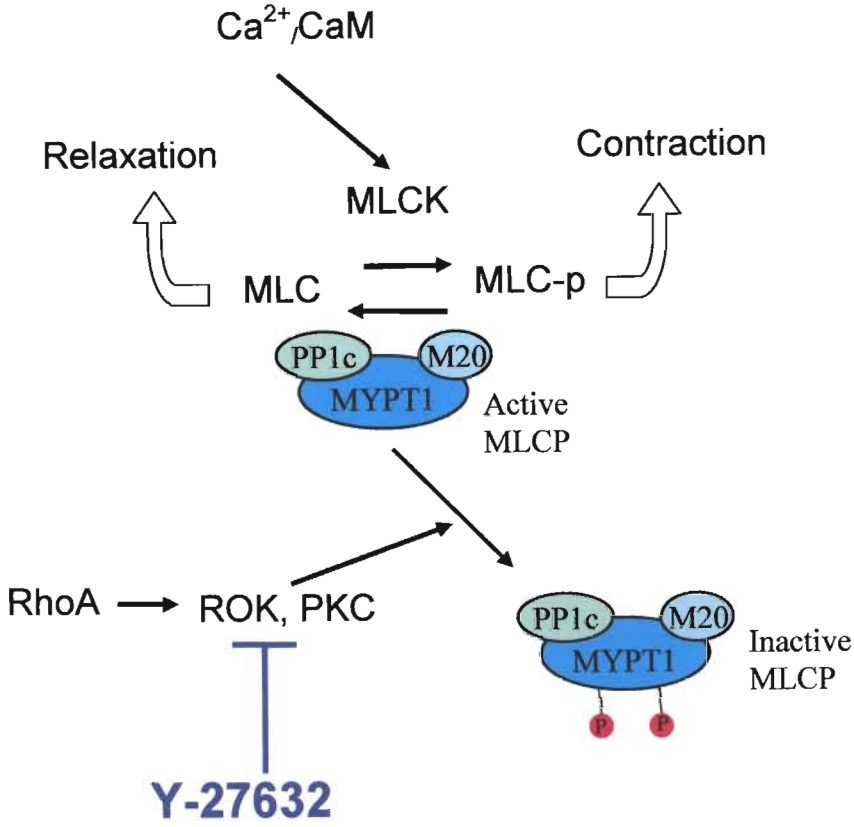
Ca2+/CaM
Relaxation
MLCK
Contraction
MLC
MLC-p
PP1c
M20
MYPT1
Active
MLCP
RhoA
ROK, PKC
PP1c
M20
MYPT1
Inactive
MLCP
P
P
Y-27632

Figure 1

calcium is desensitization. The determining factor of calcium sensitivity is the ratio of MLCK to MLCP activity. Therefore, any stimulus which increases MLCK activity in the absence of calcium changes, or decreases the rate of dephosphorylation, would result in a net increase in MLC_{20}-p (Fig1)(Somlyo et al. 1999). These two pathways of increased MLC-p result in calcium sensitization.

Smooth muscle MLCK (smMLCK) is specific for the RLC of myosin II in smooth muscle and nonmuscle cells (Gallagher et al. 1997). Smooth muscle and nonmuscle myosin II phosphorylation levels are controlled by MLCK and MLCP and thus regulate several vital physiological organ systems, cellular functions and disease states including vascular, gastrointestinal, uterine smooth muscle, angiogenesis, fibroblast contraction, cytoskeletal modeling by stress fibers, asthma, hypertension, endothelial permeability and atherosclerosis (reviewed by (Somlyo and Somlyo 2003).

Alternative splicing of the gene responsible for MLCK results in short (130-150kDa) and long (208-214kDa) isoforms of smMLCK as well as telokin, a 77-90kDa PKG substrate implicated in Ca^{2+} desensitization (Gallagher et al. 1997; Kamm and Stull 2001). Although both isotypes are present in smooth muscle cells, the long isoform dominants as its extended amino terminus convey higher affinity for actin binding than the short isoform (Blue et al. 2002).

An increase in calcium sensitivity is the result of decreased activity of MLCP. The phosphatase is a haloenzyme composed of three subunits. This tetramer consists of two regulatory subunits, and a catalytic subunit (Ito et al. 2004). The catalytic subunit is a 38 kDa type 1 protein phosphatase delta isoform, while the regulatory subunits consist

of a small 20 kDa regulatory subunit (M20) and a larger 110 kDa targeting subunit (Myosin Phosphatase targeting subunit 1, MYPT1) (Hartshorne et al. 1998). The interaction of the three subunits to form the functional enzyme is based on the numerous protein binding domains contained within the MYPT sequence, including the PP1 and M20 binding domains (reviewed by Ito et al. 2004)(see Fig 1) The PP1 binding motif occurs at amino acids 35-38, followed by seven ankyrin repeats. The c-terminus contains the M20 binding domain at amino acids 934-1006. The main regulatory phosphorylation sites are threonine 696 and 853 (human nomenclature). Thr 696 is the inhibitory phosphorylation site and thus phosphorylation at this site inhibits the phosphatase activity of the haloenzyme (Ichikawa et al. 1996). However, Thr 853 falls within the myosin binding domain on MYPT (714-933) therefore when phosphorylated at this site dissociation of the haloenzyme from myosin occurs(Velasco et al. 2002). Thus, phosphorylation at this site decreases efficiency of the enzyme by decreasing availability of the substrate. The binding domain for phosphorylated MLC occurs at 170-296 and thus binding of MLC-p reduces the Km of PP1 for its substrate (Tanaka et al. 1998).

Regulation of MLCP by MYPT occurs when either Thr 696 or 850 are phosphorylated. RhoA Kinase can phosphorylate both sites however several other kinases have also been shown to phosphorylate the inhibitory Thr 696 site such as zip kinase, zip- like kinase, p21-activated protein kinase, integrin linked kinase, raf-1 and myotonic dystrophy protein kinase (reviewed by Ito et al. 2004; Somlyo and Somlyo 2003).

Figure 2. Model of Actin/Myosin Crossbridges Activation of MLCK resulting in phosphorylation of the regulatory light chain of myosin (MLC_{20}) results in activation of myosin ATPase. Activation of myosin ATPase results in myosin interaction with Actin and attachment-detachment cycling to produce force in the direction of the blue arrows.

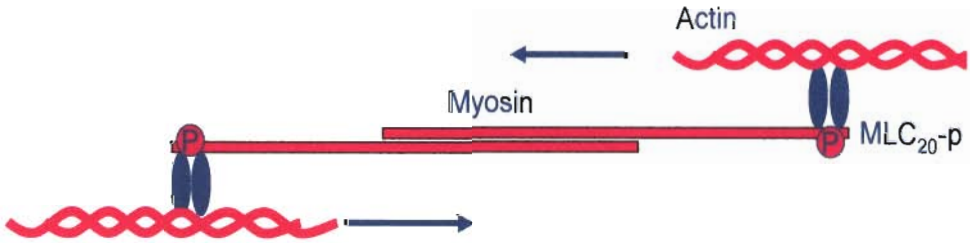

Figure 2

RhoA Kinase is a serine/threonine kinase activated by the small GTPase, RhoA. RhoA-GTP binds to the RhoA binding domain on ROK resulting in a conformational change, autophosphorylation and subsequent activation of the kinase. To understand the localization of these events within the cell, it is useful to have a basic knowledge of the regulation of GTPases. RhoA cycles between active and inactive forms based on the binding of GTP or GDP, respectively. RhoA guanine nucleotide exchange factors, or GEFs, direct the exchange of GDP for GTP and therefore activate RhoA (Olofsson 1999; Seasholtz et al. 1999). Conversely, RhoA GTPase activating proteins, or GAPs, catalyze the hydrolysis of GTP to GDP, thus inactivating RhoA (Seasholtz et al. 1999). The active form of RhoA is membrane bound due to its prenylated carboxy terminus. When inactive, RhoA-GDP is found bound to a Guanine dissociation inhibitor (GDI) protein, which functions to bind the hydrophobic carboxy terminus, thus rendering RhoA-GDP a cytosolic protein (reviewed by Schmidt and Hall 2002).

Regulation of MLCP by CPI-17 is dependent on the phosphorylation state of Thr 38 on CPI-17. CPI-17 is a 17 kDa inhibitory protein for MLCP that when phosphorylated at Thr 38 has a 1000 fold greater inhibitory effect. CPI-17 is primarily phosphorylated by protein kinase C (PKC) but in vitro may also be phosphorylated by RhoA Kinase, MYPT1 kinase, PKN, PKA, integrin linked kinase, and p21- activated protein kinase (Eto et al. 1994; Hamaguchi et al. 2000; Koyama et al. 2000; MacDonald et al. 2001; Takizawa et al. 2002; Dubois et al. 2003). Interestingly, many of these kinases also directly phosphorylate the inhibitory site of MYPT.

Calcium Desensitization and Relaxation of Contraction

Calcium Desensitization may occur by three events: (1) inhibition of calcium sensitization by inhibiting RhoA/ROK or CPI-17 dephosphorylation; (2) activation of MLCP by PKG, PKA or telokin; (3) Inhibition of MLCK by CaMKII or PAK. Early evidence for calcium desensitization includes an inhibitory serine-512 site on the carboxyl terminal end of smMLCK. Phosphorylation at this site by PKA results in a 10-fold reduction in Ca^{2+}/CaM affinity for MLCK (Conti and Adelstein 1981). On the other hand, PKG appears to induce calcium desensitization by, directly or indirectly, increasing MLCP activity and not by phosphorylating MLCK (Wu et al. 1996). However, there does appear to be cross activation between the PKA and PKG activation pathways. PKG mediated desensitization is produced by NO activating soluble guanylate cyclase to produce cGMP. PKG knockout animals have been shown to be hypertensive, demonstrating the importance of this desensitization pathway (Feil et al. 2002). The desensitizing effect of PKG by activating MLCP may not occur through direct phosphorylation of the regulatory subunit, MYPT, but through direct interaction of the leucine zipper motives of PKG and MYPT. PKG may also desensitize contraction through phosphorylation of RhoA at ser-188 to inhibit its activation of ROK (Sauzeau et al. 2000). Additionally, 8-bromo-cGMP and Forskolin, activators of PKG and PKA, respectively, phosphorylate telokin at ser-13 to induce desensitization (Wu et al. 1998; MacDonald et al. 2000), although the molecular mechanisms remain unknown. Inhibition of RhoA may also occur through PKG/PKA mediated phosphorylation events on RhoA and $G\alpha_{13}$, respectively, resulting in calcium desensitization(Sauzeau et al.

2000; Sawada et al. 2001; Manganello et al. 2003). Pharmacological inhibition of either ROK or MLCK would also result in calcium desensitization and decreased force production. In conclusion, inhibition of MLCK or MLCP inhibition pathways, or activation of MLCP would result in decreased force through calcium desensitization of myosin.

Thin Filament Regulation

Recent evidence has developed implicating thin-filament regulation in smooth muscle contraction. These include the finding that cross-bridge cycling rates can vary without changes in MLC-p and additional reports of tension and MLC-p dissociation. Thin filaments are composed of filamentous actin forming 6-8nm diameter filaments and compose 30-50% of total noncollagenous proteins (Morgan and Gangopadhyay 2001). Two important thin-filament regulatory proteins, caldesmon and calponin will be reviewed, and the mechanism by which they regulate smooth muscle contraction discussed.

Caldesmon (CaD) is a 90kDa myosin, tropomyosin, calmodulin and actin binding protein. The main functions of this binding protein are to bind actin and inhibit myosin ATPase activity. Thus, this protein has also been implicated in tethering of actin and myosin which may contribute to force maintenance due to the latch state. However, it is more like that CaD acts as an ON/OFF switch due to its inhibition of myosin ATPase. There are two myosin binding domains near the amino terminus of the peptide, while the carboxy terminus contains the actin binding site(Lee et al. 2000), the

calmodulin binding site, as well as the regulatory phosphorylation sites of ERK and p21-activated Kinase (PAK). It is proposed that the amino terminus binds myosin while the carboxy terminus binds actin and under non-stimulated conditions the inhibition of myosin ATPase provided by CaD prevents actomyosin cycling. However, upon agonist-induced signalling, it has been shown ERK and PAK can phosphorylate CaD and prevent the inhibition of myosin ATPase and thereby permit actomyosin cross-bridge cycling and thus contraction(Foster et al. 2004; Gerthoffer et al. 1996). Additionally, Ca^{2+}/Calmodulin interaction with CaD also decreases the binding of CaD to actin and thus reduces its inhibitory actions. CaD inhibits myosin ATPase somewhat by slowing the rate-limiting step, but primarily by decreasing the actin-myosin affinity since myosin and CaD compete for a binding site on actin (reviewed by Horowitz et al. 1994). Thus, smooth muscle thin-filament regulation by CaD is a result of tethering actin and myosin and inhibiting myosin phosphorylation until agonist-induced signalling phosphorylates CaD itself to abolish its inhibitory effects.

Calponin (CaP) is a 32kDa basic regulatory protein which binds actin, calmodulin, myosin, desmin, and phospholipids and functions to inhibit ATPase activity(Wills et al. 1993; Mabuchi et al. 1997; Shirinsky et al. 1992; Bogatcheva and Gusev 1995). The name for calponin is derived from '*cal*cium and *cal*modulin-binding tro*ponin* T-like protein". The structure of CaP begins at the amino terminus with a calponin homology domain (CH domain), homologous across isoforms, followed downstream by the actin binding domain which is homologous to the troponin I actin binding domain and is referred to as the TnI-like sequence. Following this sequence is a

three c-terminal repeats reported to be involved in both actin and ERK binding. CaP appears to inhibit the unloaded shortening velocity rather than affect total isometric force production (reviewed by Morgan and Gangopadhyay 2001). Therefore, whereas CaD may function as a crosslinking "on/off" switch for actomyosin interactions, it appears that CaP is involved in the modulation of velocity of contractions and has been implicated in the latch state of the muscle. CaP can be phosphorylated by two serine/threonine kinases, PKC and CaMK II, both of which phosphorylate CaP at Ser^{175} and Thr^{184}, the latter being the preferred site for PKC (Winder and Walsh 1990). Dephosphorylation of CaP occurs by a type II A protein phosphatase. In vitro studies have shown that phosphorylation of CaP results in decreased binding to actin, loss of inhibition of myosin ATPase, and loss of inhibition of unloaded shortening velocity. However, in vivo, phosphorylation studies have yet to show any data suggesting that CaP regulation by phosphorylation actually does occur. Therefore, although the mechanism of contractile regulation by CaP phosphorylation exists, it remains to be determined whether this regulatory pathway is on in "vivo". The role of CaP in the latch state of smooth muscle contraction has also lead to a working model of interaction of CaP with actin and myosin to produced tonic sustained force. It is suggested that the amino terminus of CaP interacts with phosphorylated MLC upon contraction while the carboxy terminus remains attached to actin filaments. Therefore CaP would tether actin and myosin and slow cross-bridge cycling. The latch state of smooth muscle contraction may also provide insight to the mechanisms regulating tonic versus phasic muscles. It is known the CaP and CaD compete for actin binding but CaP has a higher affinity. In

1992, Haeberle showed that phasic muscles have 3-10 times more CaD than Cap. Therefore, it may be possible that phasic muscles do not maintain force, or latch state, because their high levels of CaD displace CaP from actin and thus do not elicit the latch phenomenon.

The mechanisms by which thin-filaments regulate smooth muscle contraction include tethering of filaments, inhibition of myosin ATPase and latch-state regulation. CaD and CaP themselves can also be regulated by de/phosphorylation from other phosphatases and kinases. Smooth muscle contraction, although classically oversimplified as proportional to MLC-p levels and cross-bridge cycling rates, is now known to be much more complex and regulated by both thick and thin filament signalling systems.

The tensegrity model first proposed by Ingber in 1993, describes how the cytoskeleton is arranged such that the cell is always under a certain amount of 'prestress' determined by the delicate balance of actin filaments, microtubules (MT) and intermediate filaments(IF). In this model, actin filaments provide tension on the cell while MT's resist compression establishing a basal equilibrium. However, if this equilibrium were disrupted by a MT depolymerizing drug, tension would increase on the actin filaments due to decreased stability normally provided by the MT struts. Alterations in this tension/compression equilibrium would change the contractile response of the cell, and although integrins have been linked to MAPK activation little is known about integrin signaling directly to MT's. Depolymerization of MT's has been shown to release free Rho-GEF, a small GTPase responsible for activation of RhoA and

subsequently ROK (Krendel et al. 2002). Additionally, ROK inhibitors HA-1077 and Y-27632 have been shown to inhibit KCl-induced contractions in the presence of Colchicine in aortic artery (Zhang et al. 2001). Activation of the RhoA/ROK signaling cascade would result in inhibition of MLCP and increased MLC-p leading to increased force production.

Physiological factors that Regulate Blood Pressure

Increases in blood pressure are regulation by several physiological factors in a coordinated effort. Acute nervous reflexes occur immediately within seconds of increased pressure including baroreceptor and chemoreceptor mechanisms which are nerve endings in the walls of arteries. Additionally, the stress relaxation mechanism, a property of smooth muscle cells relaxing due to increased stretch of the vessel with pressure, decrease blood pressure. Additionally, hormonal mechanisms such as the renin-angiotensin-aldosterone system regulated by the kidney control the blood volume in the arterial and venous system to regulate blood pressure (all mechanisms reviewed by Guyton 1991). Studying the properties of smooth muscle individually of hormonal or nervous control, such as regulation of contractile responses due to increased pressure or stretch of the vessel, may lead to a better understanding of blood pressure regulation.

TRP channel inhibition by 2-APB

Transient receptor potential channels are calcium channels that have been shown to have sequence similarity with the α_1 subunit of L-type VOC (Phillips et al. 1992).

Recent data suggests TRP channels may be responsible for store-operated Ca^{2+} entry (Albert and Large 2003). Additionally, data suggests that dihydropyridine Ca^{2+} channel blockers may be highly potent inhibitors of certain SOCs (Willmott et al. 1996; Krutetskaia et al. 1997; Curtis and Scholfield 2001). Recent publications from this lab showed that 2-APB, a reported IP3 channel inhibitor (Maruyama et al. 1997), produced tonic force inhibition in KCl-induced contractions(Ratz and Berg 2006). This data agrees the recent publications suggesting that 2-APB inhibits TRP channels (Ma et al. 2000; Trebak et al. 2002; Xu et al. 2005). While it is generally accepted that α-agonists activate SOCs by depleting intracellular calcium stores (Somlyo and Somlyo 2003), we examined the activation of SOCs by KCl and determined that KCl-induced contraction is due to Ca^{2+} entry through both L-type VOCs and TRPs (Ratz and Berg 2006). We proposed that nifedipine inhibits both channel types and 2-APB inhibits only TRPs resulting in, respectively, complete and partial inhibition of KCl-induced contraction.

Chapter 2: Introduction

Vascular smooth muscle contraction is induced by increases in $[Ca^{2+}]_i$, resulting in subsequent activation of Ca^{2+}-calmodulin-dependent myosin light chain (MLC) kinase (reviewed by Kamm and Stull 1985) and increases in phosphorylation of the regulatory light chain of myosin (Himpens and Somlyo 1988; Ratz and Murphy 1987; and reviewed by Murphy 1988). KCl-induced force responses have been attributed to depolarization of the membrane and thus increased opening of VOCC (Ganitkevich and Isenberg 1991; Imaizumi et al. 1989; Morgan and Morgan 1982; and reviewed by Bolton 1979; Van Breemen et al. 1978). This model has been established and accepted due to the ability of highly selective dihydropyridines, which block L-type channel activity, to abolish KCl-induced tonic increases in $[Ca^{2+}]_i$, MLC phosphorylation and force (Meisheri et al. 1981; Moreland and Moreland 1987; Peiper et al. 1971; Takuwa et al. 1987; and reviewed by Godfraind et al. 1986; Karaki et al. 1997). Stimuli that activate G protein-coupled receptors (GPCR) are attributed to increase not only calcium influx but Ca^{2+} sensitivity of contractile proteins through activation of RhoA kinase (ROK) and protein kinase C (PKC) that inhibit MLC phosphatase activity. Conventional models do not suggest KCl induces Ca^{2+} sensitivity, however, recent data

from this lab and others show that KCl can also cause increases in Ca^{2+} sensitization (reviewed by Ratz et al. 2005) by stimulation of ROK activity that is inhibited by nifedipine (Urban et al. 2003).

The Superficial Epigastric Artery (EA) is a branch of the femoral artery originating approximately 1cm below the inguinal ligament of the hip. The branches of the epigastric artery ultimately supply blood to the abdominal wall. Although studies have been done using the epigastric artery in ischemia reperfusion models, isometric force tracings have yet to be studied. Data shown here suggest the EA has a unique regulatory mechanism, distinct from FA, which responds to the degree of cyclical stretch of the artery. This study sought to determine the signaling mechanisms responsible for the observed increase in tonic, but not peak, force when EA is stretched.

Integrins transduce cellular mechanical strain through the extracellular matrix to the intracellular cytoskeletal through undetermined mechanisms involving receptor tyrosine kinase (RTK). RTK signaling has been shown to activate PAK and MAPK pathways, specifically ERK1/2, leading to MLCK activation (Klemke et al. 1997; and reviewed by Lehoux et al. 2006). This lab has previously shown ERK1/2 phosphorylation is elevated during a KCl-induced contraction in FA, but the role of MAPK signaling remains to be determined in EA(Ratz 2001).

The tensegrity model first proposed by Ingber in 1993, describes how the cytoskeleton is arranged such that the cell is always under a certain amount of 'prestress' determined by the delicate balance of actin filaments, microtubules (MT) and intermediate filaments(IF). In this model, actin filaments provide tension on the cell

while MT's resist compression establishing a basal equilibrium. However, if this equilibrium were disrupted by a MT depolymerizing drug, tension would increase on the actin filaments due to decreased stability normally provided by the MT struts. Alterations in this tension/compression equilibrium would change the contractile response of the cell, and although integrins have been linked to PI3K activation little is known about integrin signaling directly to MT's. Depolymerization of MT's has been shown to release free Rho-GEF, a small GTPase responsible for activation of RhoA and subsequently ROK(Krendel et al. 2002). Activation of the RhoA/ROK signaling cascade would result in inhibition of MLCP and increased MLC-p leading to increased force production. Therefore, this study tested the hypothesis that increased tonic-to-peak ratios observed in EA with stretch are a produce of integrin signaling through two mechanisms: 1) integrin linked PI3K activation and 2) integrin signaling resulting in MT depolymerization through an undetermined mechanism.

Chapter 3: Significance

Ca^{2+} sensitivity produced by RhoA/ROK activity has been shown to be involved in the pathophysiology of several vascular and non-vascular smooth muscle diseases as well as normal physiological processes (reviewed by Somlyo, 2003). VSM diseases involving an increase in ROK activity include hypertension and vasospasm of both coronary and cerebral blood vessels (Uehata, 1997; Chrissobolis, 2001). Inhibition of ROK activity by the selective inhibitor, Y-27632, has been shown to reduce blood pressure in three experimental models (Uehata et al. 1997). This research will give a better understanding of the mechanisms regulating ROK induced Ca^{2+} sensitivity in vascular smooth muscle.

Chapter 4: Methods

Tissue preparation

Tissues were prepared as described previously (Ratz 1993). Femoral and epigastric arteries from adult female New Zealand White rabbits were cleaned of adhering tissue and stored in cold (0-4°C) physiological saline solution: PSS, composition in mM: NaCl, 140; KCl, 4.7; $MgSO_4$, 1.2; $CaCl_2$, 1.6; Na_2HPO_4, 1.2; morpholino-propanesulfonic acid, 2.0 (adjusted to pH 7.4 at either 0°C or 37°C, as appropriate); Na_2ethylenediamine tetraacetic acid (EDTA; to chelate trace heavy metals), 0.02; and D-glucose, 5.6. High purity (greater than 17 MΩ distilled and deionized) water was used throughout. The endothelium of each artery was removed by gently rubbing the intimal surface with a metal rod, and Femoral tissues were cut into artery rings ~3-4 mm wide while epigastric tissues where cut ~1-2mm wide for force measurements and ~5-6mm wide for phosphorylation measurements. Each muscle ring was secured between a micrometer for length adjustments and isometric force transducer (model 52, Harvard Apparatus, South Natick, MA) in a water-jacketed muscle chamber (Radnoti Glass Technology, Inc., Monrovia, CA). In one set of force experiments shown in Fig 5, strips of rabbit urinary bladder (detrusor) free from underlying urothelium were isolated as described previously (Shenfeld et al. 1998), cleaned of overlying fat and serosal connective tissues, and secured between tissue clips

for force measurements. Isometric contractions were measured as described previously (Ratz 1993). Voltage signals from force transducers were digitized (CIO-DAS16F, ComputerBoards, Middleboro, MA), visualized on a computer screen as force (g), and stored by software command to a hard disk for later analyses (see Fig 3). All data analyses were accomplished using DasyLab (DasyTec, Amherst, NH) and an electronic spread sheet.

Isometric force

Contractile force (F) was measured as described previously (Ratz 1993). Tissues were allowed to equilibrate at 37°C for one hour, and the muscle length at which active force was maximum (L_o) was then determined for each tissue with K^+ as agonist (110 mM KCl substituted isosmotically for NaCl) using an abbreviated length-tension curve (Herlihy and Murphy 1973; Ratz 1993). Once tissues were stretched to L_o, no further length changes were imposed except in experiments where the effect of stretch or slack length was tested (ie. 1.2Lo and 0.7Lo). For each pre-loaded tissue, the degree of steady-state F produced at L_o by incubation for 5-10 min in 110 mM KCl was equal to the optimal force for muscle contraction (F_o), and subsequent contractions were calculated as F/F_o. Arteries contracted with KCl were incubated with 1μM phentolamine to block potential α-adrenergic receptor activation caused by release of norepinephrine from peri-arterial nerves. In experiments designed to activate α-adrenergic receptors, 10 μM phenylephrine (PE) was used because this concentration produces a strong, sustained contraction in rabbit arteries (Ratz 1993).

Figure 3. Model of Force Measurements Diagram depicting mechanical measurement of force in both Femoral and Epigastric artery. These measurements were digitized using an A/D board and analyzed with a computer software package.

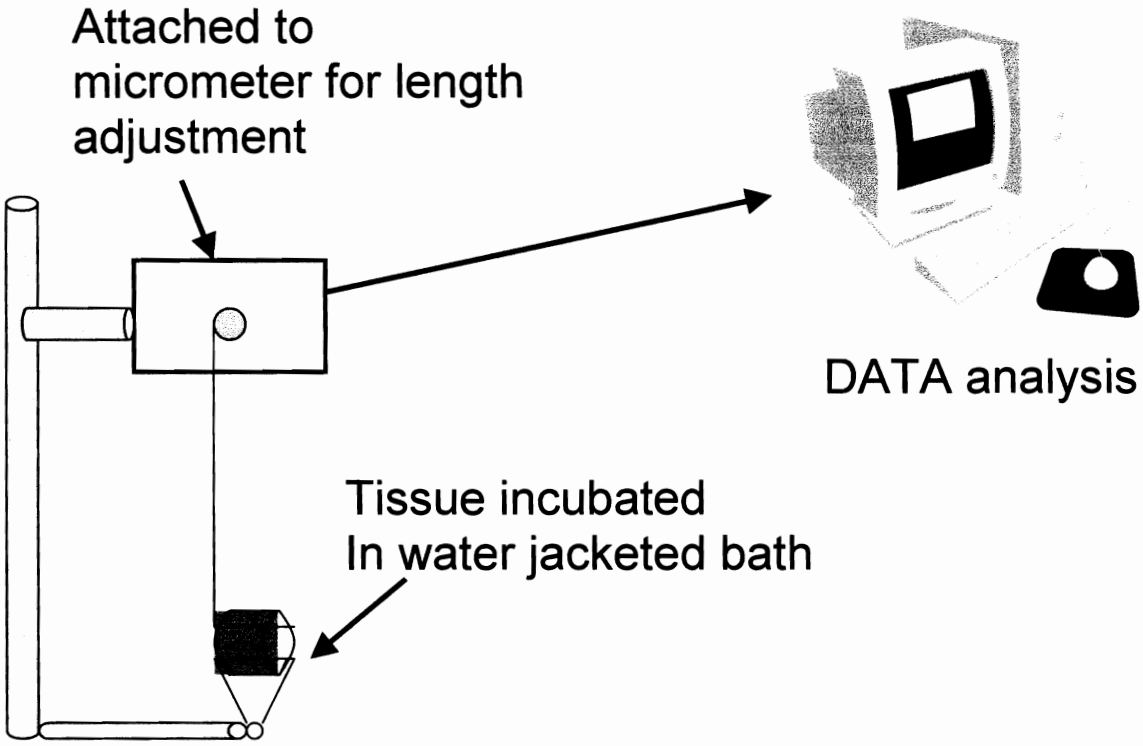
Attached to
micrometer for length
adjustment
DATA analysis
Tissue incubated
In water jacketed bath

Figure 3

Myosin light chain phosphorylation

The degree of MLC phosphorylation was measured as described previously (Ratz and Murphy 1987; Ratz 1993). Arteries were quick-frozen in an acetone-dry ice slurry, slowly warmed to room temperature, dried, weighed and homogenized on ice in 8M urea, 2% Triton X-100 and 20 mM dithiothreitol. Isoelectric variants of the 20 kDa myosin light chains (MLCs) were separated by 2-D (IEF/SDS) PAGE followed by Western Blot, visualized by colloidal gold stain, and the relative amounts of phosphorylated and non-phosphorylated MLCs were quantified by digital image analysis.

Intracellular Free Calcium ($[Ca^{2+}]_i$) Measurement

$[Ca^{2+}]_i$ was measured as described previously with minor modifications (Ratz 1993). Arterial rings were secured in a temperature-controlled myograph suitable for simultaneous measurement of force and fluorescence (Danish Myo Technology, Denmark) which was positioned on the stage of an Olympus IX71 inverted microscope with attachments to a Deltaram V fluorometer and photomultiplier tube (Photon Technology Internation, Lawrenceville, NJ). Tissues were maintained at 37°C and loaded for 2.5 hours with the 7.5 μM of the Ca^{2+} indicator, Fura-2(PE3)/AM (Teflabs, Austin, TX) with 0.01% Pluronic F-127 added to enhance solubility. Tissues were washed for 30 minutes prior to the start of the experiment to remove extracellular Fura-2(PE3)/AM. Fluorescence emissions at 510 nm were collected by a photomultiplier tube in response to alternate excitation at 340 and 380 nm with a 75-W xenon lamp (Photon

Technologies International). At the end of the experiment, background tissue fluorescence was recorded by adding 4 mM $MnCl^2$ plus 10 μM ionomycin to quench the fura-2 signal. $[Ca^{2+}]_i$ was presented as the fluorescence ratio (F^{340}/F^{380}) corrected for background fluorescence. To reduce tissue-to-tissue variability for statistical comparisons, $[Ca^{2+}]_i$ for each tissue was reported as the fraction of that produced at 2 minutes of a KCl stimulus and the minimum value produced during exposure of tissues to a Ca^{2+}-free solution containing 1 mM EGTA plus 10 μM ionomycin.

Drugs

Nifedipine, Colchicine and Taxol were obtained from Sigma; GF-109203X from Alexis; Y-27632, cytochalasin, latrunculin and H-1152 from EMD biosciences and Wortmannin from Axxora. Y-27632 and H-1152 were dissolved in water. Nifedipine, Colchicine and latrunculin were dissolved in ethanol. Wortmannin, cytochalasin and Taxol were dissolved in DMSO. Ethanol or DMSO was added at final concentration no greater than 0.1% that had no effect on stimulus-induced responses.

Statistics

The null hypothesis was examined using Student's t-test (when 2 groups were compared) or using a one-way Analysis of Variance (ANOVA). To determine differences between groups following ANOVA, the Student-Neuman-Keuls post-hoc test was used. In all cases, the null hypothesis was rejected at $P < 0.05$. For each study

described, the *n* value was equal to the number of rabbits from which arteries were obtained.

Chapter 5: Results

Effect of cyclic stretch on KCl-induced contraction in EA

A KCl-induced contraction of the epigastric artery consists of two phases, a rapid peak in force production followed by slow, sustained tonic force. Increased circumferential stretch of the artery to 1.2 times its optimal length (1.2 Lo) resulted in an increase in tonic force with minimal increase in peak force compared to 0.7 and 1.0 Lo (Fig 4A). Similarly, decreases in stretch resulted in suppressed tonic-to-peak ratios. Thus, as epigastric artery was lengthened, the ratio obtained from tonic to peak force values increased (Fig 4C). The femoral artery, the large conduit artery from which epigastric artery originates, did not show length dependent regulation of tonic force upon KCl-induced stimulation (Fig 4 D&E; force tracing Fig 5). However, tonic force development was delayed by 23 seconds in 1.2 Lo compared to 0.7 Lo FA (Fig 5-inset). These results support the hypothesis that epigastric artery displays a unique regulatory mechanism dependant on stretch for tonic force maintenance distinct from the femoral artery.

ROK involvement in tonic force maintenance

To establish whether increased tonic force due to increased ROK activity at longer lengths played a role in stretch-induced tonic force maintenance we tested the

Figure 4. Effect of cyclic stretch on KCl-induced contraction in EA Force tracing (a) of EA contracted at 1.0, 1.2 and 0.7Lo. Individual peak and tonic force data for EA and FA (B&D, respectively); and tonic-to-peak ratios of EA and FA (C&E, respectively). Note EA tonic-to-peak ratios increased with increasing stretch of the vessel while FA did not. Data in panel C -F are mean ± SE of peak or tonic data normalized to the peak KCl-induced responses, n = 8; * $P < 0.05$ compared to basal values.

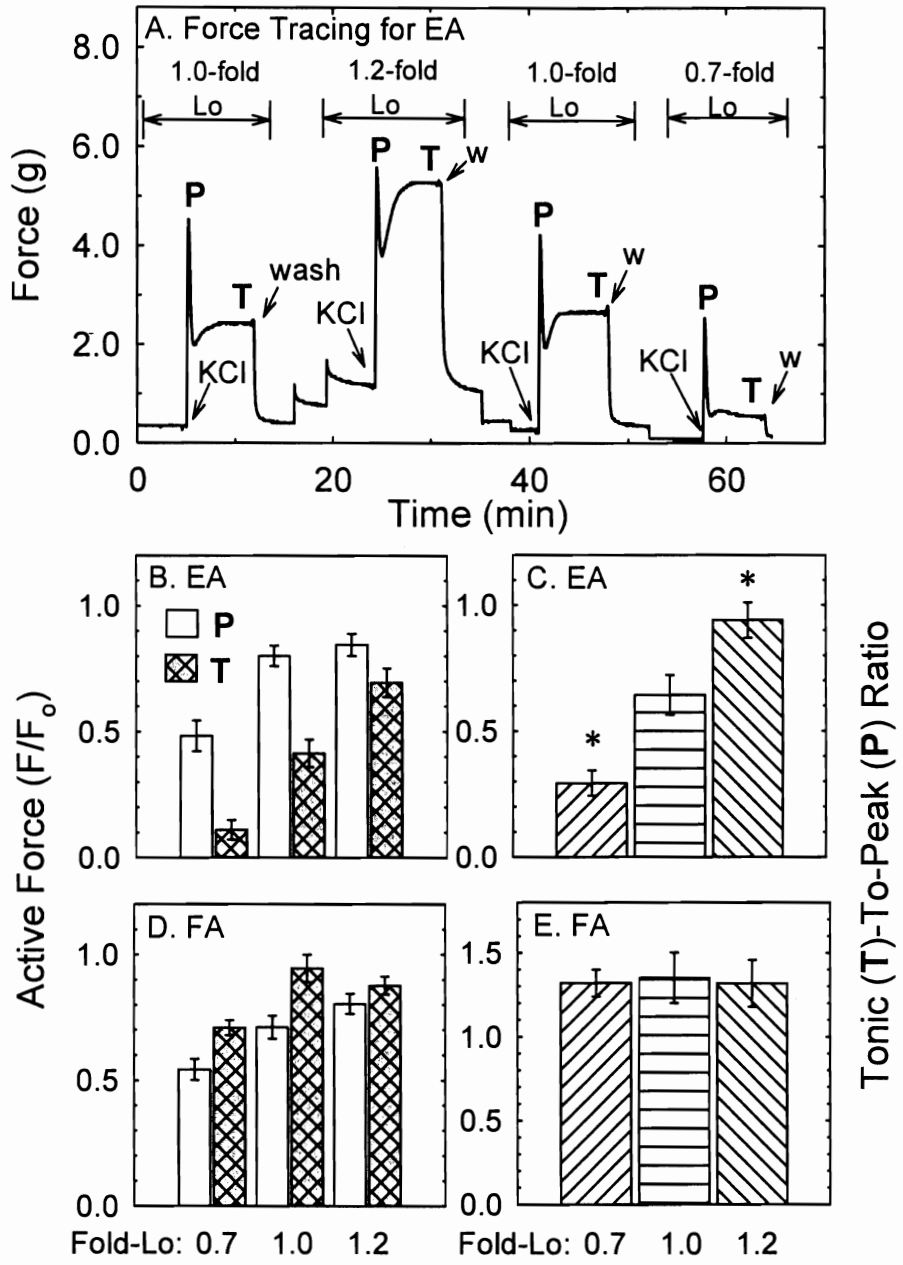
A. Force Tracing for EA
1.0-fold
1.2-fold
1.0-fold
0.7-fold
Lo
Force (g)
Time (min)
P
T
w
wash
KCl
B. EA
C. EA
D. FA
E. FA
Active Force (F/F$_o$)
Tonic (T)-To-Peak (P) Ratio
Fold-Lo: 0.7 1.0 1.2
Fold-Lo: 0.7 1.0 1.2

Figure 4

Figure 5. Effect of cyclic stretch on KCl-induced contraction in FA Force tracing of FA contracted at 0.7 and 1.2 Lo. Inset of peak contractions lined-up shows time delay to tonic force. Data is mean ± SE of peak or tonic data normalized to the peak KCl-induced responses, n = 7.

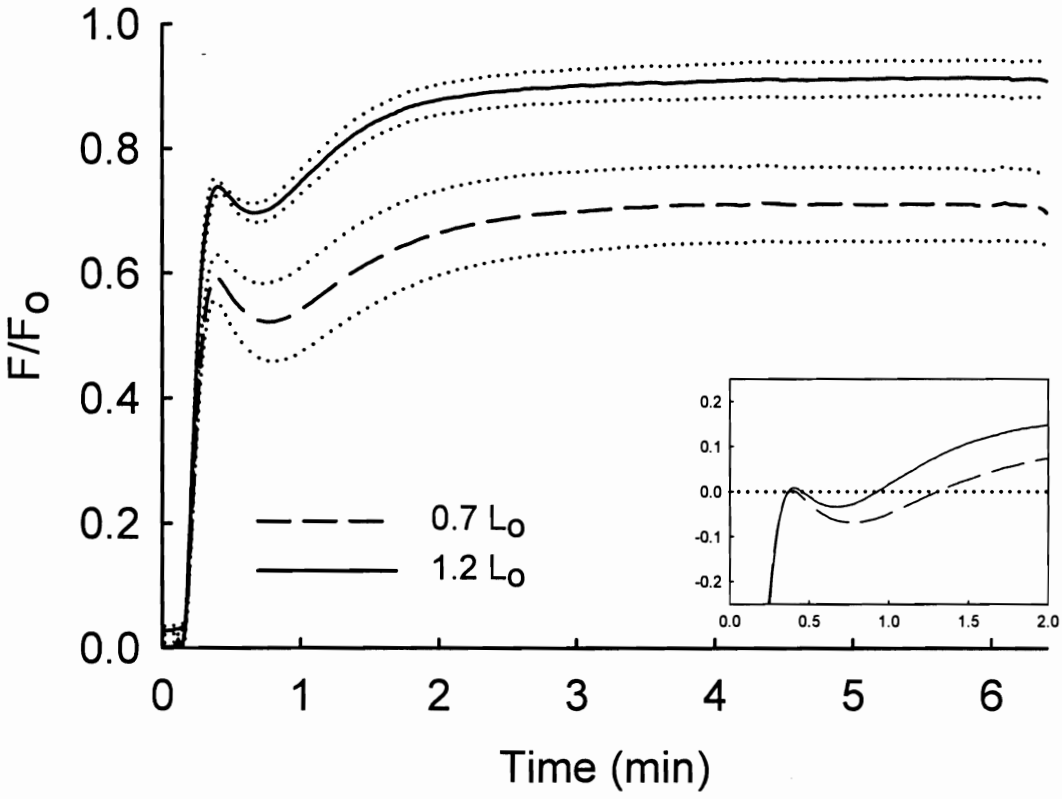
1.0
0.8
0.6
0.4
0.2
0.0
F/Fo
0.7 Lo
1.2 Lo
0
1
2
3
4
5
6
Time (min)
0.2
0.1
0.0
-0.1
-0.2
0.0
0.5
1.0
1.5
2.0

Figure 5

involvement of ROK in KCl-induced contractions. Y-27632 (1μM) was incubated in tissues at 0.7 Lo, 1.0 Lo and 1.2 Lo prior to contraction with KCl (Fig 6A). These data indicate tonic force was inhibited at all lengths by an IC50 concentration, the dose required to inhibit contraction by half, of the inhibitor but peak force was minimally inhibited at longer lengths. A dose response curve was performed adding Y-27632 to the tonic phase of a KCl-induced contraction at all lengths (Fig 6b). These results show the efficacy and potency of Y-27632 does not change with length, suggesting that ROK activity is increased with length and is the major regulator of tonic force at all lengths and additional signaling pathways are not activated with length changes. However, to ensure PKC was not also participating in force regulation, the PKC(n,c) inhibitor, GF-109203X, was used. Pre-incubation of GF-109203X did not inhibit peak or tonic force of KCl-induced contraction at all lengths except at the 1.2Lo tonic time point (Fig 6c). These data suggest that ROK contributes to KCl-induced tonic force maintenance at all lengths while PKC appears to play a role only at 1.2 Lo. Treatment with nifedipine (1μM), an L-type calcium channel blocker, resulted in both peak and tonic force inhibition at all lengths (data not shown).

Cytosolic calcium response to increased muscle stretch

To determine whether increases in $[Ca^{2+}]$ lead to increased force due to increased MLC-p, tissues were loaded with the fluorescent calcium indicator, Fura-2. Although unstimulated muscle tone of the muscle increased with stretch (Fig 7A), basal

Figure 6. Effect Y-27632 and GF-109203X on KCl-induced contraction in EA

Tonic-to-peak ratios and dose response curve of EA incubated with the ROK inhibitor Y-27632 (A&B, n=3&4, respectively). Tonic-to-peak ratios of EA incubated with the PKC inhibitor GF-109203X (c, n=7). Data in panel C -F are mean ± SE of peak or tonic data normalized to the peak KCl-induced responses represented by dotted line at 100%, * $P < 0.05$ compared to control values.

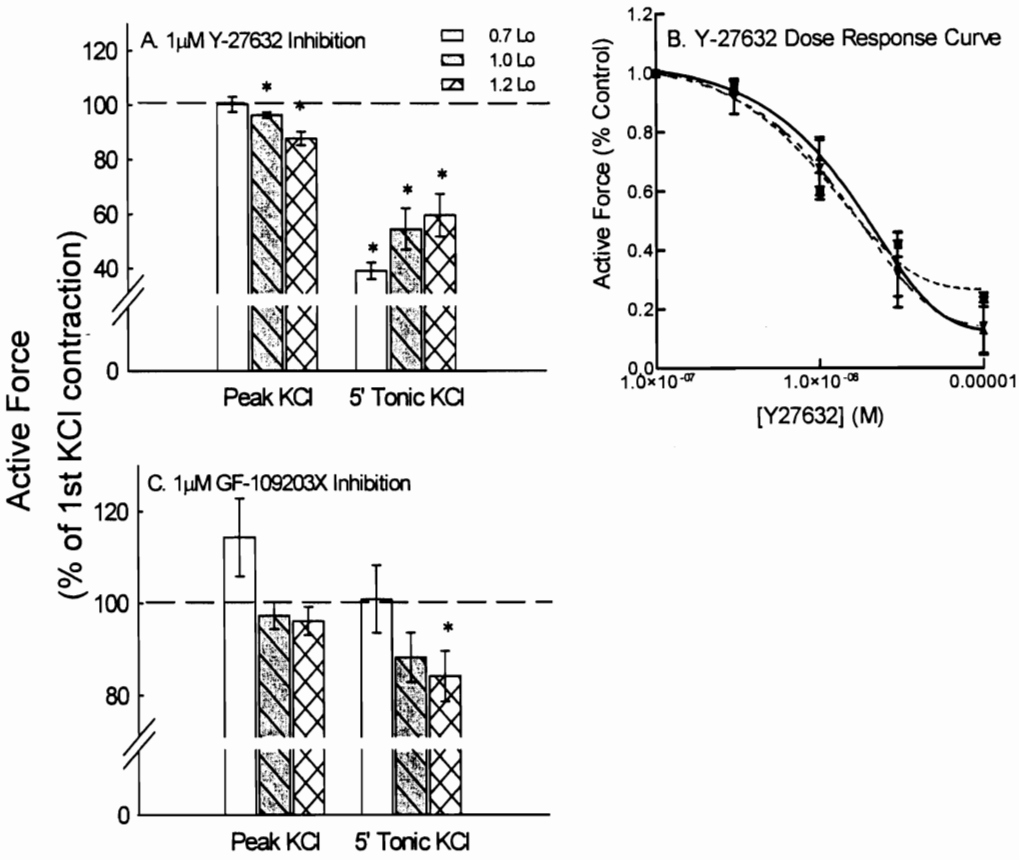
A. 1μM Y-27632 Inhibition
0.7 Lo
1.0 Lo
1.2 Lo
Active Force
(% of 1st KCl contraction)
120
100
80
60
40
0
Peak KCl
5' Tonic KCl
B. Y-27632 Dose Response Curve
Active Force (% Control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1.0×10⁻⁰⁷
1.0×10⁻⁰⁵
0.00001
[Y27632] (M)
C. 1μM GF-109203X Inhibition
120
100
80
0
Peak KCl
5' Tonic KCl

Figure 6

$[Ca^{2+}]$ levels were not altered either by increasing or decreasing the length of the muscle (Fig 7b). Therefore, basal $[Ca^{2+}]$ was not altered by length changes and was not the source of additional force production basally or upon KCl stimulation. In addition, KCl-induced depolarization produce equal $[Ca^{2+}]$ increases at all lengths (data not shown), further suggesting that increases in $[Ca^{2+}]$ either basally or in response to KCl are not responsible for sustained tonic force with stretch.

Effect of 10μM Colchicine on KCl-induced contractile force in EA

To determine whether depolymerization of microtubules increased tonic force maintenance by increasing free RhoGEF, tissues at 0.7Lo were first contracted with KCl, followed by incubation with 10μM Colchicine for one hour. Subsequent responses to KCl after Colchicine incubation were compared to control contractions prior to Colchicine treatment (Fig 8a). Those tissues incubated with Colchicine showed a significant increase in tonic to peak ratio of 206% ±10 of initial KCl stimulation. Control tissues stimulated with KCl after incubation with vehicle showed a 136% ± 12 increase above initial stimulation (Fig 8c). These data suggest 10μM Colchicine increases tonic force maintenance as measured by tonic-to-peak ratios compared to vehicle control tissues at 0.7Lo. Treatment with Taxol (1μM), a microtubule stabilizer, did not increase tonic-to- peak ratios in agreement with previous publication (Zhang et al. 2000). Furthermore, when the specific ROK inhibitor H-1153 (0.3μM) was added to tissues along with Colchicine, tonic-to-peak ratio increases were significantly reduced

Figure 7. Cytosolic calcium response to increased muscle stretch Response of unstimulated EA to length changes. Basal tone increased in response to stretch of EA (a). Cytosolic calcium as measured by FURA-2 imaging shoed no increase in intracellular calcium in response to stretch. Data are mean ± SE of basal data normalized to the peak KCl-induced responses, n = 5; * $P < 0.05$ compared to 1.0 Lo values; Ψ $P<0.05$ compared to 0.7Lo values.

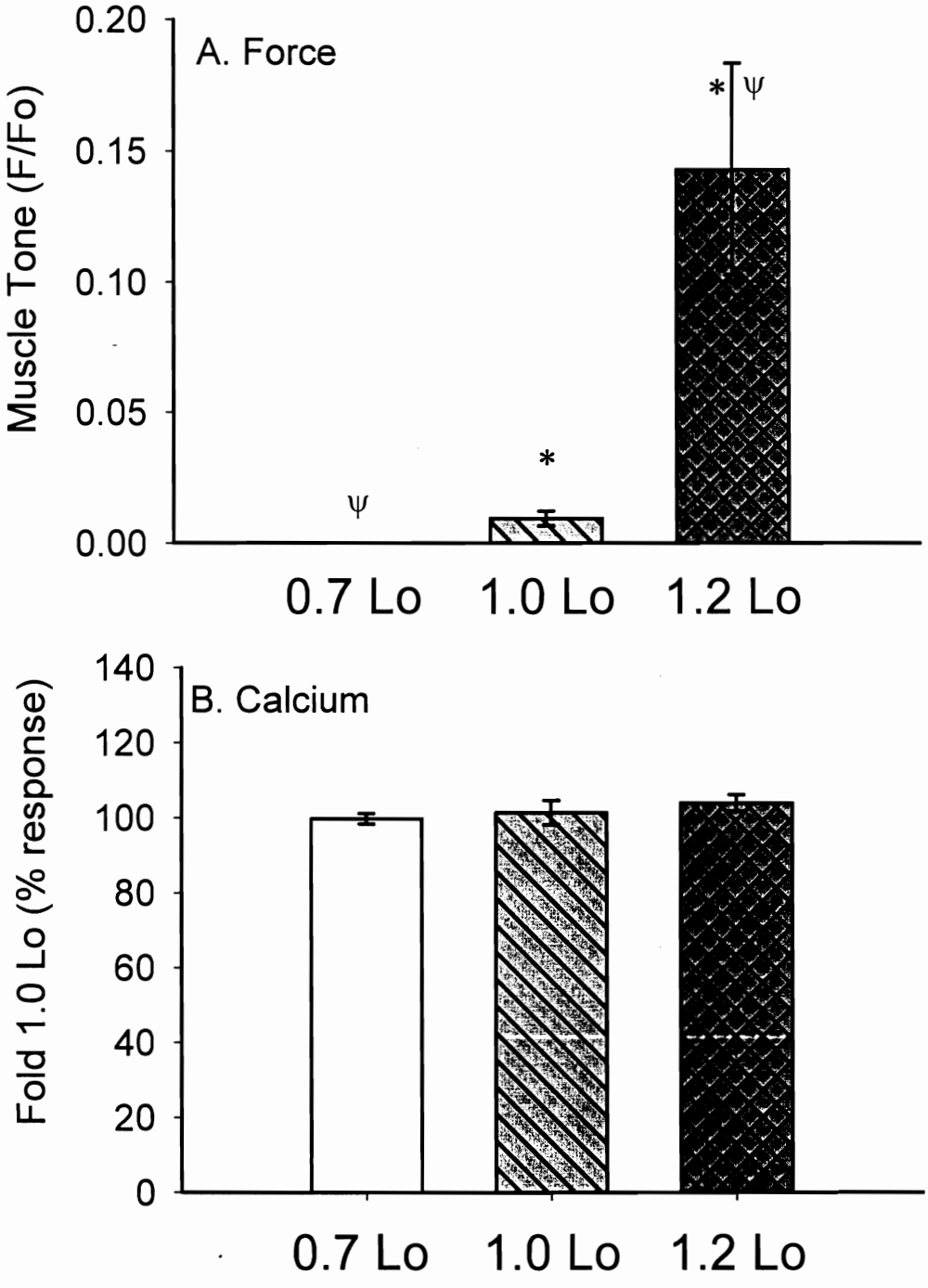
A. Force
Muscle Tone (F/Fo)
0.20
0.15
0.10
0.05
0.00
*
ψ
*
ψ
0.7 Lo
1.0 Lo
1.2 Lo
B. Calcium
Fold 1.0 Lo (% response)
140
120
100
80
60
40
20
0
0.7 Lo
1.0 Lo
1.2 Lo

Figure 7

to approximately 50% below Colchicine alone (Fib 8b). Thus, tonic force maintenance can be increased by microtubule depolymerization in 0.7 Lo tissues and increased tonic-to-peak ratios are dependent on ROK mediated signaling mechanisms. Agents that can regulate actin polymerization state such as Latrunculin, which depolymerizes actin, decreased ratios at both 0.7 and 1.2 Lo. This represents the artery dependency on actin for production of contractile force. These data taken together support the hypothesis that RhoGEF is located on microtubules and upon MT depolymerization RhoGEF becomes released into the cytosol to activate the RhoA/ROK pathway.

Myosin Light Chain$_{20}$ phosphorylation levels

To compare the length regulation of epigastric artery to the tonic femoral artery, MLC_{20}-p was measured at 0.7 Lo and 1.2 Lo at basal and 5' KCl-induced stimulation. Femoral artery showed no significant difference between 0.7 Lo and 1.2 Lo basal MLC_{20}-p levels (18.20±2.64 and 17.34±3.99 for 0.7 Lo and 1.2 Lo, respectively). Stimulated phosphorylation levels in femoral artery also were not significantly different between 0.7 Lo and 1.2 Lo (43.49±2.40 and 46.04±1.62, respectively). On the contrary, epigastric artery stretch to 1.2 Lo showed significantly increased levels of MLC_{20}-p compared to 0.7 Lo at both unstimulated conditions (9.40±1.71 and 16.70±3.42 for 0.7 Lo and 1.2 Lo, respectively) and KCl-stimulated tissues (27.78±2.23 and 34.15±1.48 for 0.7 Lo and 1.2 Lo, respectively) (Fig 9a-b). Incubation of 3μM Wortmannin, a PI3 kinase and MLCK inhibitor, and $0Ca^{2+}$ solution resulted in decreased MLC-p in stretched but not unstretched tissues (~51% inhibition vs. ~7% inhibition,

respectively)(Fig 9c). These data suggest PI3K or MLCK is activated under stretched conditions when the tissue is not stimulated with KCl. Additionally, incubation of 0.7 Lo tissues with Colchicine resulted in an increase in MLC-p, although not significant. The ROK inhibitor, H-1152, significantly reduced MLC-p induced by Colchicine below basal while Y-27632 did not alter control MLC-p (Fig 9D) or force (data not shown).

Figure 8. Effect of 10μM Colchicine on KCl-induced contractile force in EA Force tracing (a) of EA at 0.7Lo incubated for 1hr. with 10μM Colchicine. Note that KCl-induced tonic force is increased after Colchicine incubation. Inhibition of tonic force increases due to Colchicine by the ROK inhibitor H-1152 and not the PKC inhibitor GF-109203X (b). Pharmacological agents effecting the polymerization stat of microtubule and actin increase or decrease tonic force compared to peak (tonic-to-peak ratios) in a KCl-induced contraction in EA (c and d). Data are mean ± SE of peak or tonic data normalized to the peak KCl-induced responses, n = 3-5; * $P < 0.05$ compared to control values, Ψ represents inhibition of both peak and tonic force such that Tonic-to-peak ratios were not altered.

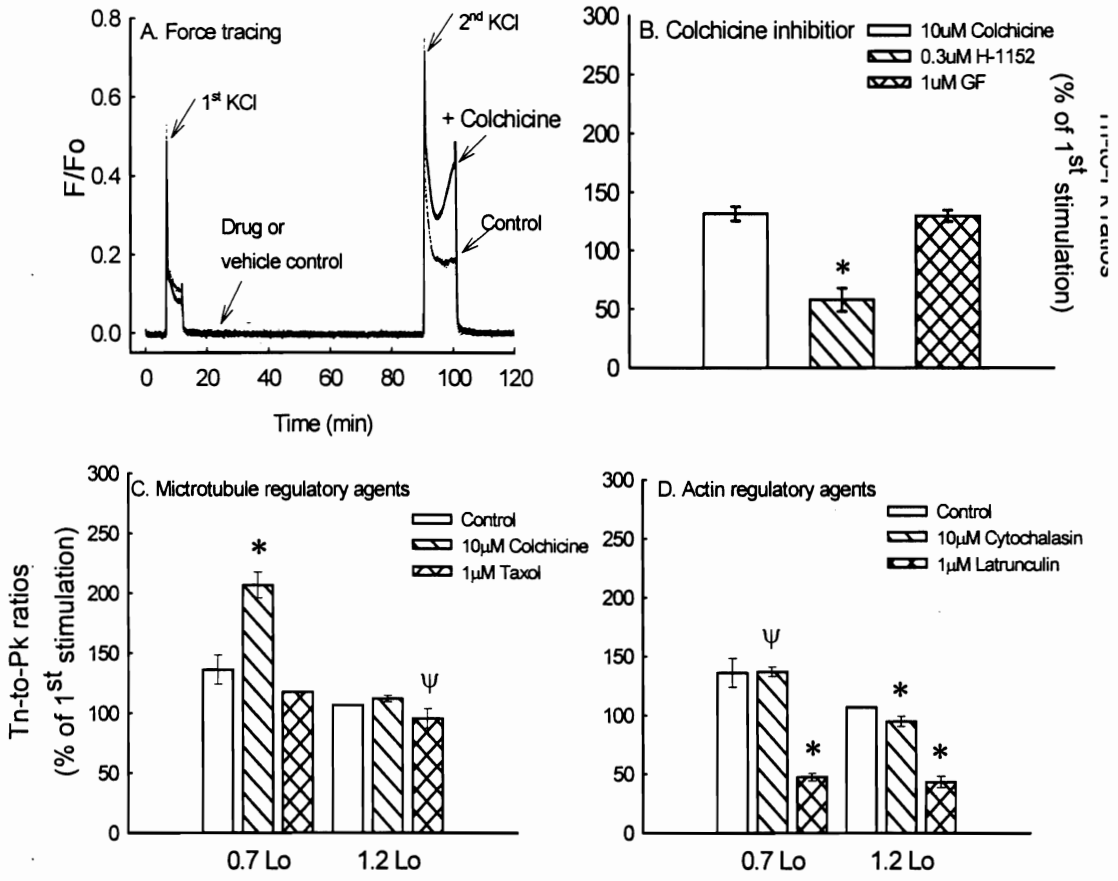
A. Force tracing
1st KCl
2nd KCl
+ Colchicine
Control
Drug or
vehicle control
F/Fo
Time (min)
B. Colchicine inhibitior
10uM Colchicine
0.3uM H-1152
1uM GF
(% of 1st stimulation)
C. Mictrotubule regulatory agents
Control
10μM Colchicine
1μM Taxol
Tn-to-Pk ratios
(% of 1st stimulation)
0.7 Lo
1.2 Lo
D. Actin regulatory agents
Control
10μM Cytochalasin
1μM Latrunculin
0.7 Lo
1.2 Lo

Figure 8

Figure 9. Myosin Light Chain$_{20}$ phosphorylation levels MLC_{20}-p of 0.7 and 1.2Lo tissue samples of EA and FA (a & b). Note that MLC-p increases basally with stretch in EA but not FA, however KCl-induced increases with stretch in both EA and FA are modest. Incubation of 0.7 and 1.2 Lo tissues with $0Ca^{2+}$ and 3mM Wortmannin, a MLCK inhibitor, results in a decrease in basal MLC-p of 1.2Lo but not 1.2 Lo tissues (c). Basal incubation of colchicine results in increased MLC-p (although not significant) which is decreased by co-incubation with H-1152 (d). However Y-27632 does not decrease MLC-p in basal tissues not incubated with Colchicine. Data in panels a & b are mean ± SE of percent MLC20 phosphorylation, while data in panels c & d are taken as a fraction of basal control. n = 5-12; * $P < 0.05$ compared to basal values.

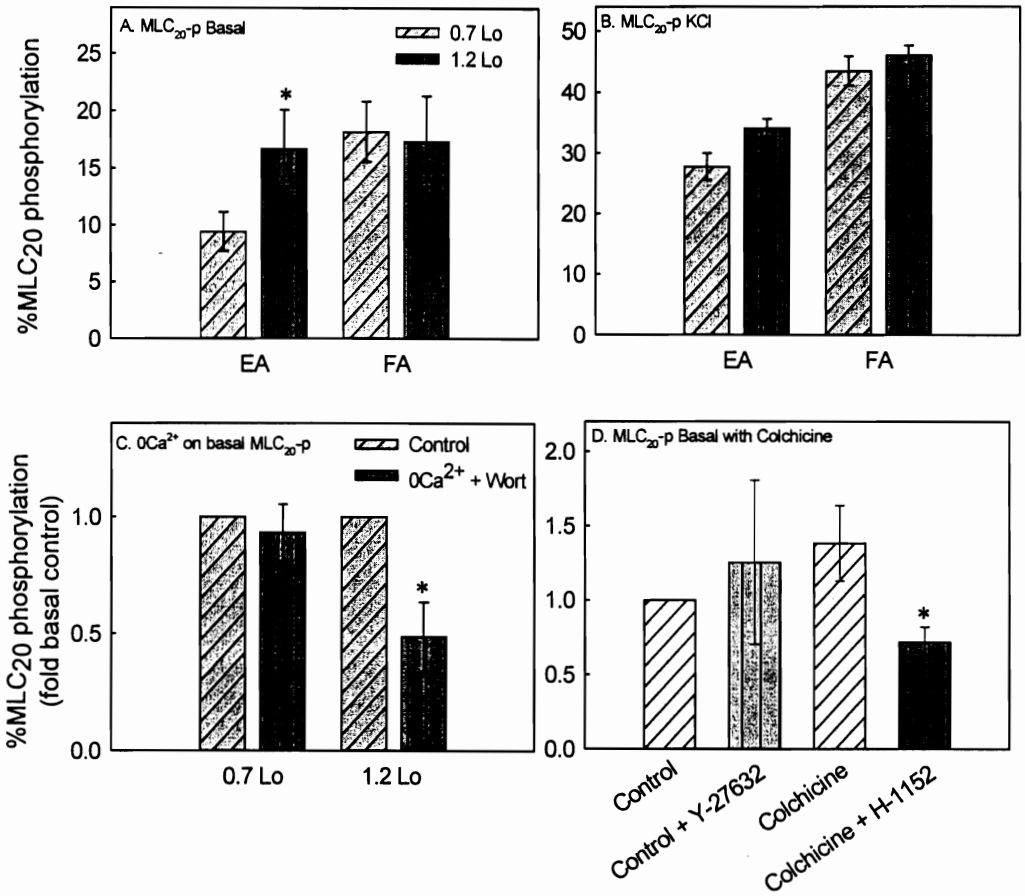
A. MLC20-p Basal
0.7 Lo
1.2 Lo
%MLC20 phosphorylation
25
20
15
10
5
0
*
EA
FA
B. MLC20-p KCl
50
40
30
20
10
0
EA
FA
C. 0Ca2+ on basal MLC20-p
Control
0Ca2+ + Wort
%MLC20 phosphorylation (fold basal control)
1.0
0.5
0.0
*
0.7 Lo
1.2 Lo
D. MLC20-p Basal with Colchicine
2.0
1.5
1.0
0.5
0.0
*
Control
Control + Y-27632
Colchicine
Colchicine + H-1152

Figure 9

Chapter 6: Discussion

Vascular smooth muscle contraction of both rabbit femoral and epigastric artery results in a biphasic force in response to KCl stimulation. In both arteries, the initial rapid peak phase is induced by rapid increases in intracellular calcium through VOC and subsequent MLCK activation. The slow sustained tonic phase is due to increased calcium sensitivity of crossbridges by inhibition of MLCP. Thus, two main mechanisms are responsible for a KCl-induced contraction in both FA and EA. However, EA has a unique stretch dependent regulatory mechanism not seen in FA. The objective of this study was to identify the signaling mechanisms responsible for stretch dependant tonic force maintenance in EA.

FA tonic force is mainly regulated by ROK activation. Increased ROK activity results in decreased MLCP by phosphorylation of the regulatory subunit and thus preventing phosphatase activity. Previous studies from this lab have shown a dose dependency of tonic force on ROK inhibition (Urban et al. 2003). Furthermore, KCl contractions induce ROK translocation to intracellular signalosomes named caveolae. This translocation is dependant on intracellular calcium influx during contraction. These data suggest that ROK activation is also calcium dependant. Although it is not clear what mechanisms ROK uses to translocate to the membrane, it is known that ROK is

colocalized with caveolae after 30seconds of KCl exposure in both FA and EA (EA is preliminary data n=1).

The aim of the current study initially was to investigate the hypothesis that stretch-dependant tonic force maintenance was due to an increase in calcium through SAC resulting in increased ROK and MLCK activity and subsequent MLC-p. The elevated tonic force maintenance observed, induced by increased preload of the tissue, was distinct from the femoral artery. Blood vessels are exposed to external mechanical forces in the form of cyclic mechanical strain and shear stress due to blood pressure and blood flow. Our data shows stretch of results in increased muscle tone and corresponding MLC_{20}-p. These basal unstimulated increases were not inhibited by ROK or PKC inhibitors, but were inhibited by the PI3K, MLCK and MAPK inhibitor, Wortmannin (Ferby et al. 1994; Nakanishi et al. 1992). Additional data in KCl-stimulated EA showed increased tonic-to-peak ratios in stretched versus unstretched muscle. Tonic force at all lengths was inhibited by the ROK inhibitor, Y-27632, while the PKC inhibitor only inhibited stretched tonic force under KCl stimulated conditions. Our data also showed $[Ca^{2+}]_i$ did not increase with increasing muscle stretch, demonstrating that stretch operated calcium channels were not participating in this response. Thus, the initial hypothesis that SAC channels were responsible for increased MLC-p and tonic force maintenance was proved invalid.

The revised hypothesis was to test whether stretch resulted (1) integrin linked MAPK activation of MLCK and (2) MT depolymerization and subsequent RhoA activation through release of free RhoGEF. Pharmacologic depolymerization of MTs by

Colchicine in unstretched EA resulted in increased KCl-stimulated tonic-to-Peak ratios, similar to control stretched ratios. The increase in tonic-to-peak ratios in response to MT depolymerization seen in unstretched tissues was also inhibited by a ROK inhibitor and not a PKC inhibitor.

The role of integrins in the transduction of mechanical strain from the extracellular matrix to the intracellular cytoskeletal signaling has been shown to activate receptor tyrosine kinases (RTK), PAK and MAPK pathways, specifically ERK1/2, leading to MLCK activation (Klemke et al. 1997; and reviewed by Lehoux et al. 2006). A proposed model involving the activation of MLCK or PI3K phosphorylation of MLC through integrin signaling may explain the observed results from stretch induced increases in tonic force. Furthermore, previous studies by this lab in FA have shown stretch in FA to 1.3Lo induces ERK phosphorylation. These results support the hypothesis that stretch may activate MAPK or directly activate MLCK. However, preliminary studies using the MAPK inhibitor, U-0126, showed no decrease in basal tone. Interestingly, the MAPK inhibitor did decrease peak force without effecting tonic force in 1.2 Lo tissues but had not effect on either peak or tonic force at 0.7 Lo. Theses data suggest MAPK activation plays a role in the phasic portion of the contraction classically attributed to calcium entry. However, basal tone does not seem to be a product of MAPK activity. Thus, further studies are needed to determine which other kinases which may activate MLCK under stretched conditions. Potential kinases which phosphorylate MLC directly are PI3K, Zip kinase and Integrin linked kinase. PI3 kinase is activated by receptor tyrosine kinase phosphorylated by integrin activation.

Wortmannin inhibited stretch induced increases in MLCp, thus PI3 Kinase would be a likely candidate for integrin linked signaling (Fig 10-model).

According to the tensegrity model proposed by Ingber (Ingber 1993), MTs, actin microfilaments (MFs), and intermediate filaments (IFs) are arranged in the cytosol in such a way that MTs resist compression while MFs and IFs resist tension. However, if MTs are depolymerized, the result is increased tension on the extracellular matrix due to a disruption in the balance of compressional and tensile forces. Although the tensegrity model clearly presents a role of MT's to resist compression during muscle contraction, it has not been determined whether stretch alters MT stability in arterial smooth muscle. However, increased stretch of the rat brachial plexus has been shown to result in decreased MT's (Kikukawa et al. 2003) suggesting increased stretch does depolymerize MT's. The present study did not demonstrate decreased MT's due to stretch directly; however, increased tonic force was observed in unstretched tissues treated with the MT depolymerizing agent Colchicine. Furthermore, several RhoGEF proteins have been found to interact with MTs, and are released when MT are depolymerized (Ren et al. 1998; van Horck et al. 2001). RhoA has also been shown to be involved in the assembly of focal adhesions and subsequent phosphorylation of FAK in VSM cells (Carbajal and Schaeffer 1999). These previously published results may provide a model of KCl-activation of ROK due to increased mechanical stretch. Our data suggests depolymerization due to Colchicine increases MLC-p which is inhibited by H-1152, suggesting a ROK mediated mechanism is responsible for the increase in MLC-p. However, tissues stretched to 1.2 Lo and incubated with the ROK inhibitor, Y-27632,

do not display a decrease in tone or MLC-p. Conflicting data may be explained by the ability of the transducers to sense such small alterations in force. Another possibility is that the relative changes in MT density when stretched to 1.2Lo are not comparable to pharmacological depolymerization with Colchicine.

In conclusion, mechanical stretch of EA results basally in increased MLC20-p to cause an increase in muscle tone. The pathway through which unstimulated stretch induces MLC-p was not studied in this report and remains to be elucidated but appears not to involve MAPK activation. However, it does appear that MAPK pathways are activated during contraction in stretched, but not unstretched tissues. Additionally, our data supports pharmacological MT depolymerization activates ROK signaling pathways. Upon KCl-stimulation ROK translocates to caveolae to become activated by RhoA-GTP and induces calcium sensitivity. This study characterizes a unique regulatory pathway in which the degree of cyclic mechanical stretch increases tonic force relative to peak. The ability to modulate tonic force as a function of stretch could potentially serve as a feedback mechanism in vascular smooth muscle diseases such as hypertension and hypertrophy. Modulation of the cytoskeletal organization may prove to be a useful clinical treatment for many diseases and acute syndromes of VSM and further studies are needed to clarify the role of MT reorganization in VSM contraction.

Figure 10. Proposed model of Stretch-induced basal and tonic force Data presented has shown increases in force in the absence of stimulus are inhibited by a PI3K inhibitor. It is not yet known what mechanism activates the PI3K pathway, but integrin and subsequent RTK activation may be a potential mechanism which warrants further studies. Additionally, although stretch alone was not sensitive to ROK inhibitor, inducible tonic force maintenance using MT depolymerizing drugs was sensitive to ROK inhibitors. Thus, further studies are needed to determine if stretch alters actin or MT polymerization states to increase tonic force during stimulation.

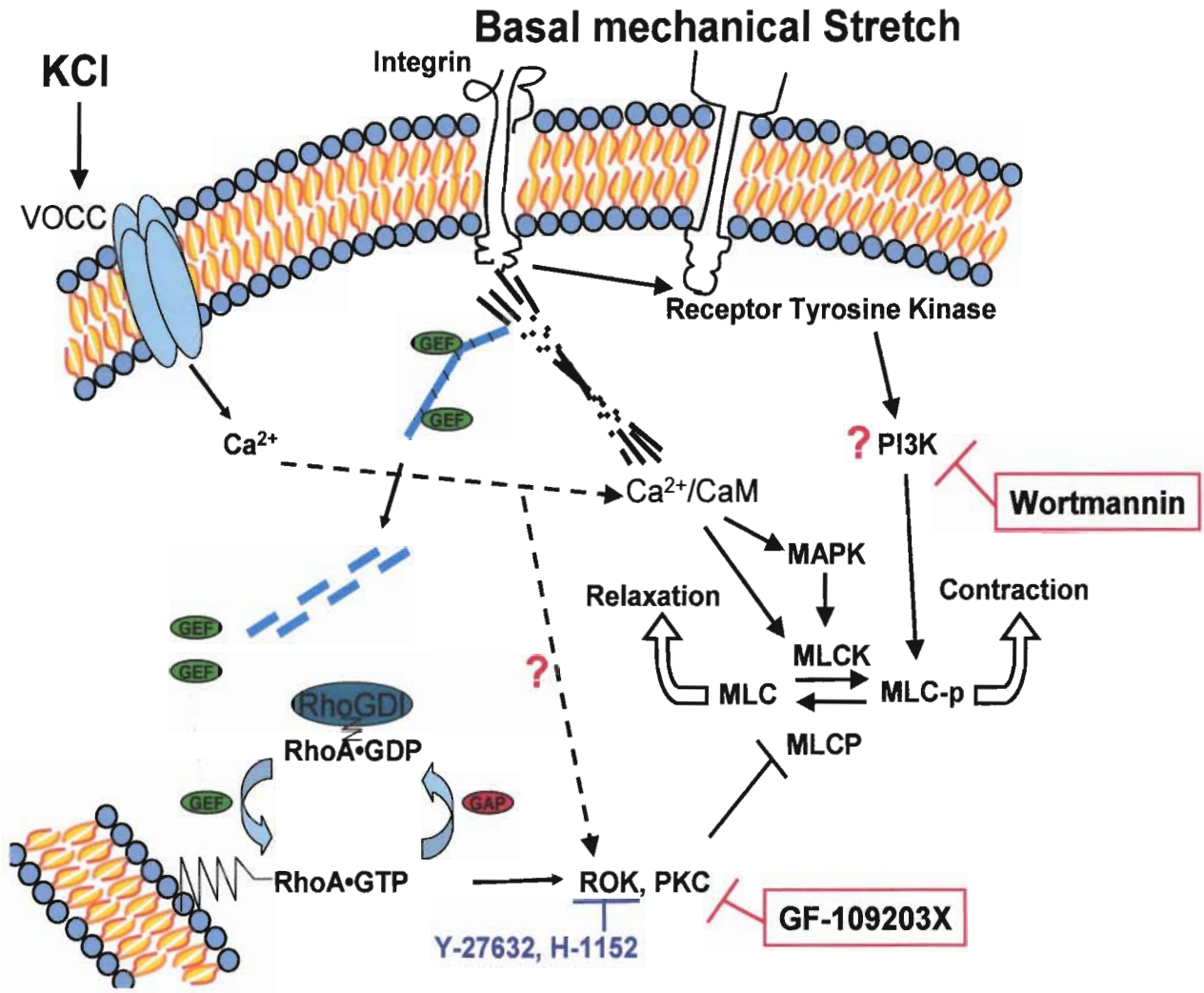
Basal mechanical Stretch
KCl
Integrin
VOCC
Receptor Tyrosine Kinase
GEF
GEF
Ca2+
? PI3K
Wortmannin
Ca2+/CaM
MAPK
Relaxation
Contraction
GEF
GEF
?
MLCK
MLC
MLC-p
RhoGDI
RhoA•GDP
MLCP
GEF
GAP
RhoA•GTP
ROK, PKC
GF-109203X
Y-27632, H-1152

Figure 10

Literature Cited

Literature Cited

Albert AP and Large WA. Store-operated Ca2+-permeable non-selective cation channels in smooth muscle cells. *Cell Calcium* 33: 345-356, 2003.

Blue EK, Goeckeler ZM, Jin Y, Hou L, Dixon SA, Herring BP, Wysolmerski RB and Gallagher PJ. 220- and 130-kDa MLCKs have distinct tissue distributions and intracellular localization patterns. *Am J Physiol Cell Physiol* 282: C451-460, 2002.

Bogatcheva NV and Gusev NB. Interaction of smooth muscle calponin with phospholipids. *FEBS Lett* 371: 123-126, 1995.

Bolton TB. Mechanisms of action of transmitters and other substances on smooth muscle. *Physiol Rev* 59: 606-718, 1979.

Carbajal JM and Schaeffer RC, Jr. RhoA inactivation enhances endothelial barrier function. *Am J Physiol* 277: C955-964, 1999.

Conti MA and Adelstein RS. The relationship between calmodulin binding and phosphorylation of smooth muscle myosin kinase by the catalytic subunit of 3':5' cAMP-dependent protein kinase. *J Biol Chem* 256: 3178-3181, 1981.

Curtis TM and Scholfield CN. Nifedipine blocks Ca^{2+} store refilling through a pathway not involving L-type Ca^{2+} channels in rabbit arteriolar smooth muscle. *J Physiol* 532: 609-623, 2001.

Dillon PF, Aksoy MO, Driska SP and Murphy RA. Myosin phosphorylation and the cross-bridge cycle in arterial smooth muscle. *Science* 211: 495-497, 1981.

Dubois T, Howell S, Zemlickova E, Learmonth M, Cronshaw A and Aitken A. Novel in vitro and in vivo phosphorylation sites on protein phosphatase 1 inhibitor CPI-17. *Biochem Biophys Res Commun* 302: 186-192, 2003.

Eddinger TJ and Murphy RA. Developmental changes in actin and myosin heavy chain isoform expression in smooth muscle. *Arch Biochem Biophys* 284: 232-237, 1991.

Eddinger TJ and Murphy RA. Two smooth muscle myosin heavy chains differ in their light meromyosin fragment. *Biochemistry* 27: 3807-3811, 1988.

Eto M, Isonishi K, Fukui Y, Morita F, Nishi N and Tokura S. Inhibition of acto-myosin subfragment-1 ATPase activity by peptides corresponding to various segments of the 20-kDa domain of myosin heavy chain. *J Biochem (Tokyo)* 115: 701-707, 1994.

Feil R, Gappa N, Rutz M, Schlossmann J, Rose CR, Konnerth A, Brummer S, Kuhbandner S and Hofmann F. Functional reconstitution of vascular smooth muscle cells with cGMP-dependent protein kinase I isoforms. *Circ Res* 90: 1080-1086, 2002.

Ferby IM, Waga I, Sakanaka C, Kume K and Shimizu T. Wortmannin inhibits mitogen-activated protein kinase activation induced by platelet-activating factor in guinea pig neutrophils. *J Biol Chem* 269: 30485-30488, 1994.

Foster DB, Huang R, Hatch V, Craig R, Graceffa P, Lehman W and Wang CL. Modes of caldesmon binding to actin: sites of caldesmon contact and modulation of interactions by phosphorylation. *J Biol Chem* 279: 53387-53394, 2004.

Gallagher PJ, Herring BP and Stull JT. Myosin light chain kinases. *J Muscle Res Cell Motil* 18: 1-16, 1997.

Ganitkevich VY and Isenberg G. Depolarization-mediated intracellular calcium transients in isolated smooth muscle cells of guinea-pig urinary bladder. *J Physiol* 435: 187-205, 1991.

Gerthoffer WT, Yamboliev IA, Shearer M, Pohl J, Haynes R, Dang S, Sato K and Sellers JR. Activation of MAP kinases and phosphorylation of caldesmon in canine colonic smooth muscle. *J Physiol* 495: 597-609, 1996.

Godfraind T, Miller R and Wibo M. Calcium antagonism and calcium entry blockade. *Pharmacol Rev* 38: 321-416, 1986.

Guyton AC. Blood pressure control--special role of the kidneys and body fluids. *Science* 252: 1813-1816, 1991.

Hai CM and Murphy RA. Cross-bridge phosphorylation and regulation of latch state in smooth muscle. *Am J Physiol* 254: C99-106, 1988.

Hamaguchi T, Ito M, Feng J, Seko T, Koyama M, Machida H, Takase K, Amano M, Kaibuchi K, Hartshorne DJ and Nakano T. Phosphorylation of CPI-17, an inhibitor of myosin phosphatase, by protein kinase N. *Biochem Biophys Res Commun* 274: 825-830, 2000.

Hartshorne DJ, Ito M and Erdodi F. Myosin light chain phosphatase: Subunit composition, interactions and regulation. *J Muscle Res Cell Motil* 19: 325-341, 1998.

Hasegawa Y and Morita F. Role of 17-kDa essential light chain isoforms of aorta smooth muscle myosin. *J Biochem (Tokyo)* 111: 804-809, 1992.

Herlihy JT and Murphy RA. Length-tension relationship of smooth muscle of the hog carotid artery. *Circ Res* 33: 257-283, 1973.

Himpens B and Somlyo AP. Free-calcium and force transients during depolarization and pharmacomechanical coupling in guinea-pig smooth muscle. *J Physiol* 395: 507-530, 1988.

Horowitz A, Trybus KM, Bowman DS and Fay FS. Antibodies probe for folded monomeric myosin in relaxed and contracted smooth muscle. *J Cell Biol* 126: 1195-1200, 1994.

Ichikawa K, Ito M and Hartshorne DJ. Phosphorylation of the large subunit of myosin phosphatase and inhibition of phosphatase activity. *J Biol Chem* 271: 4733-4740, 1996.

Imaizumi Y, Muraki K, Takeda M and Watanabe M. Measurement and simulation of noninactivating Ca current in smooth muscle cells. *Am J Physiol* 256: C880-885, 1989.

Ingber DE. Cellular tensegrity: defining new rules of biological design that govern the cytoskeleton. *J Cell Sci* 104 (Pt 3): 613-627, 1993.

Ito M, Nakano T, Erdodi F and Hartshorne DJ. Myosin phosphatase: structure, regulation and function. *Mol Cell Biochem* 259: 197-209, 2004.

Kamm KE and Stull JT. Dedicated myosin light chain kinases with diverse cellular functions. *J Biol Chem* 276: 4527-4530, 2001.

Kamm KE and Stull JT. The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. *Annu Rev Pharmacol Toxicol* 25: 593-620, 1985.

Karaki H, Ozaki H, Hori M, Mitsui-Saito M, Amano K, Harada K, Miyamoto S, Nakazawa H, Won KJ and Sato K. Calcium movements, distribution, and functions in smooth muscle. *Pharmacol Rev* 49: 157-230, 1997.

Kelly CA, Takahashi M, Yu JH and Adelstein RS. An insert of seven amino acids confers enzymatic differences between smooth muscle myosins from the intestines and vasculature. *J Biol Chem* 268: 12848-12854, 1993.

Kikukawa K, Fukunaga K, Kato T, Yamaga M, Miyamoto E and Takagi K. Acute changes in the axonal cytoskeleton after mild stretching of the rat brachial plexus. *J Orthop Res* 21: 359-364, 2003.

Klemke RL, Cai S, Giannini AL, Gallagher PJ, de Lanerolle P and Cheresh DA. Regulation of cell motility by mitogen-activated protein kinase. *J Cell Biol* 137: 481-492, 1997.

Koyama M, Ito M, Feng J, Seko T, Shiraki K, Takase K, Hartshorne DJ and Nakano T. Phosphorylation of CPI-17, an inhibitory phosphoprotein of smooth muscle myosin phosphatase, by Rho-kinase. *FEBS Lett* 475: 197-200, 2000.

Krendel M, Zenke FT and Bokoch GM. Nucleotide exchange factor GEF-H1 mediates cross-talk between microtubules and the actin cytoskeleton. *Nat Cell Biol* 4: 294-301, 2002.

Krutetskaia ZI, Lebedev OE, Krutetskaia NI and Petrova TV. Organic and inorganic blockers of potential-dependent Ca2+ channels inhibit store-dependent entry of Ca2+ into rat peritoneal macrophages. *Tsitologiia* 39: 1131-1141, 1997.

Lee YH, Gallant C, Guo H, Li Y, Wang CA and Morgan KG. Regulation of vascular smooth muscle tone by N-terminal region of caldesmon. Possible role of tethering actin to myosin. *J Biol Chem* 275: 3213-3220, 2000.

Lehoux S, Castier Y and Tedgui A. Molecular mechanisms of the vascular responses to haemodynamic forces. *J Intern Med* 259: 381-392, 2006.

Ma HT, Patterson RL, van Rossum DB, Birnbaumer L, Mikoshiba K and Gill DL. Requirement of the inositol trisphosphate receptor for activation of store-operated Ca2+ channels. *Science* 287: 1647-1651, 2000.

Mabuchi K, Li B, Ip W and Tao T. Association of calponin with desmin intermediate filaments. *J Biol Chem* 272: 22662-22666, 1997.

MacDonald JA, Eto M, Borman MA, Brautigan DL and Haystead TA. Dual Ser and Thr phosphorylation of CPI-17, an inhibitor of myosin phosphatase, by MYPT-associated kinase. *FEBS Lett* 493: 91-94, 2001.

MacDonald JA, Walker LA, Nakamoto RK, Gorenne I, Somlyo AV, Somlyo AP and Haystead TA. Phosphorylation of telokin by cyclic nucleotide kinases and the identification of in vivo phosphorylation sites in smooth muscle. *FEBS Lett* 479: 83-88, 2000.

Malmqvist U and Arner A. Correlation between isoform composition of the 17 kDa myosin light chain and maximal shortening velocity in smooth muscle. *Pflugers Arch* 418: 523-530, 1991.

Manganello JM, Huang JS, Kozasa T, Voyno-Yasenetskaya TA and Le Breton GC. Protein kinase A-mediated phosphorylation of the Galpha13 switch I region alters

the Galphabetagamma13-G protein-coupled receptor complex and inhibits Rho activation. *J Biol Chem* 278: 124-130, 2003.

Maruyama T, Kanaji T, Nakade S, Kanno T and Mikoshiba K. 2APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of Ins(1,4,5)P3-induced Ca2+ release. *J Biochem (Tokyo)* 122: 498-505, 1997.

Meer DP and Eddinger TJ. Expression of smooth muscle myosin heavy chains and unloaded shortening in single smooth muscle cells. *Am J Physiol* 273: C1259-1266, 1997.

Meisheri KD, Hwang O and van Breemen C. Evidence for two separated Ca2+ pathways in smooth muscle plasmalemma. *J Membr Biol* 59: 19-25, 1981.

Moreland S and Moreland RS. Effects of dihydropyridines on stress, myosin phosphorylation, and V0 in smooth muscle. *Am J Physiol Heart Circ Physiol* 252: H1049-1058, 1987.

Morgan JP and Morgan KG. Vascular smooth muscle: the first recorded Ca2+ transients. *Pflugers Archiv - European Journal of Physiology* 395: 75-77, 1982.

Morgan KG and Gangopadhyay SS. Signal transduction in smooth muscle invited review: Cross-bridge regulation by thin filament-associated proteins. *J Appl Physiol* 91: 953-962, 2001.

Murphy R. Muscle Cells of Hollow Organs. *News Physiol Sci* 3: 124-128, 1988.

Nakanishi S, Kakita S, Takahashi I, Kawahara K, Tsukuda E, Sano T, Yamada K, Yoshida M, Kase H, Matsuda Y and et al. Wortmannin, a microbial product inhibitor of myosin light chain kinase. *J Biol Chem* 267: 2157-2163, 1992.

Olofsson B. Rho guanine dissociation inhibitors: pivotal molecules in cellular signalling. *Cell Signal* 11: 545-554, 1999.

Paul RJ. Smooth muscle energetics and theories of cross-bridge regulation. *Am J Physiol* 258: C369-375, 1990.

Peiper U, Griebel L and Wende W. Activation of vascular smooth muscle of rat aorta by noradrenaline and depolarization: two different mechanisms. *Pflugers Arch* 330: 74-89, 1971.

Phillips AM, Bull A and Kelly LE. Identification of a Drosophila gene encoding a calmodulin-binding protein with homology to the trp phototransduction gene. *Neuron* 8: 631-642, 1992.

Ratz PH. High α_1-adrenergic receptor occupancy decreases relaxing potency of nifedipine by increasing myosin light chain phosphorylation. *Circ Res* 72: 1308-1316, 1993.

Ratz PH. Regulation of ERK phosphorylation in differentiated arterial muscle of the rabbit. *Am J Physiol* 281: H114-H123, 2001.

Ratz PH and Berg KM. 2-Aminoethoxydiphenyl borate inhibits KCl-induced vascular smooth muscle contraction. *Eur J Pharmacol* 541: 177-183, 2006.

Ratz PH, Berg KM, Urban NH and Miner AS. Regulation of smooth muscle calcium sensitivity: KCl as a calcium-sensitizing stimulus. *Am J Physiol Cell Physiol* 288: C769-783, 2005.

Ratz PH and Murphy RA. Contributions of intracellular and extracellular Ca^{2+} pools to activation of myosin phosphorylation and stress in swine carotid media. *Circ Res* 60: 410-421, 1987.

Ren Y, Li R, Zheng Y and Busch H. Cloning and characterization of GEF-H1, a microtubule-associated guanine nucleotide exchange factor for Rac and Rho GTPases. *J Biol Chem* 273: 34954-34960, 1998.

Sauzeau V, Le Jeune H, Cario-Toumaniantz C, Smolenski A, Lohmann SM, Bertoglio J, Chardin P, Pacaud P and Loirand G. Cyclic GMP-dependent protein kinase signaling pathway inhibits RhoA-induced Ca2+ sensitization of contraction in vascular smooth muscle. *Journal of Biological Chemistry* 275: 21722-21729, 2000.

Sawada N, Itoh H, Yamashita J, Doi K, Inoue M, Masatsugu K, Fukunaga Y, Sakaguchi S, Sone M, Yamahara K, Yurugi T and Nakao K. cGMP-dependent protein kinase phosphorylates and inactivates RhoA. *Biochem Biophys Res Commun* 280: 798-805, 2001.

Schmidt A and Hall A. Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. *Genes Dev* 16: 1587-1609, 2002.

Seasholtz TM, Majumdar M and Brown JH. Rho as a mediator of G protein-coupled receptor signaling. *Molec Pharmacol* 55: 949-956, 1999.

Shenfeld OZ, Morgan CW and Ratz PH. Bethanechol activates a post-receptor negative feedback mechanism in rabbit urinary bladder smooth muscle. *J Urol* 159: 252-257, 1998.

Shirinsky VP, Biryukov KG, Hettasch JM and Sellers JR. Inhibition of the relative movement of actin and myosin by caldesmon and calponin. *J Biol Chem* 267: 15886-15892, 1992.

Somlyo AP and Somlyo AV. Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev* 83: 1325-1358, 2003.

Somlyo AP, Wu X, Walker LA and Somlyo AV. Pharmacomechanical coupling: the role of calcium, G-proteins, kinases and phosphatases. *Rev Physiol Biochem Pharmacol* 134: 201-234, 1999.

Somlyo AV and Somlyo AP. Electromechanical and pharmacomechanical coupling in vascular smooth muscle. *J Pharmacol Exp Ther* 159: 129-145, 1968.

Takizawa N, Koga Y and Ikebe M. Phosphorylation of CPI17 and myosin binding subunit of type 1 protein phosphatase by p21-activated kinase. *Biochem Biophys Res Commun* 297: 773-778, 2002.

Takuwa Y, Takuwa N and Rasmussen H. Measurement of cytoplasmic free Ca2+ concentration in bovine tracheal smooth muscle using aequorin. *Am J Physiol* 253: C817-827, 1987.

Tanaka J, Ito M, Feng J, Ichikawa K, Hamaguchi T, Nakamura M, Hartshorne DJ and Nakano T. Interaction of myosin phosphatase target subunit 1 with the catalytic subunit of type 1 protein phosphatase. *Biochemistry* 37: 16697-16703, 1998.

Trebak M, Bird GS, McKay RR and Putney JW, Jr. Comparison of human TRPC3 channels in receptor-activated and store-operated modes. Differential sensitivity to channel blockers suggests fundamental differences in channel composition. *J Biol Chem* 277: 21617-21623, 2002.

Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa H, Yamagami K, Inui J, Maekawa M and Narumiya S. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* 389: 990-994, 1997.

Urban NH, Berg KM and Ratz PH. K+ depolarization induces RhoA kinase translocation to caveolae and Ca2+ sensitization of arterial muscle. *Am J Physiol Cell Physiol* 285: C1377-1385, 2003.

Van Breemen C, Aaronson P and Loutzenhiser R. Sodium-calcium interactions in mammalian smooth muscle. *Pharmacol Rev* 30: 167-208, 1978.

van Horck FP, Ahmadian MR, Haeusler LC, Moolenaar WH and Kranenburg O. Characterization of p190RhoGEF, a RhoA-specific guanine nucleotide exchange factor that interacts with microtubules. *J Biol Chem* 276: 4948-4956, 2001.

Velasco G, Armstrong C, Morrice N, Frame S and Cohen P. Phosphorylation of the regulatory subunit of smooth muscle protein phosphatase 1M at Thr850 induces its dissociation from myosin. *FEBS Letters* 527: 101-104, 2002.

Walsh MP. Calmodulin and the regulation of smooth muscle contraction. *Mol Cell Biochem* 135: 21-41, 1994.

Willmott NJ, Choudhury Q and Flower RJ. Functional importance of the dihydropyridine-sensitive, yet voltage-insensitive store-operated Ca2+ influx of U937 cells. *FEBS Lett* 394: 159-164, 1996.

Wills FL, McCubbin WD and Kay CM. Characterization of the smooth muscle calponin and calmodulin complex. *Biochemistry* 32: 2321-2328, 1993.

Winder SJ and Walsh MP. Smooth muscle calponin: inhibition of actomyosin MgATPase and regulation by phosphorylation. *J Biol Chem* 265: 10148-10155, 1990.

Wingard CJ, Paul RJ and Murphy RA. Energetic cost of activation processes during contraction of swine arterial smooth muscle. *J Physiol* 501 (Pt 1): 213-223, 1997.

Wu X, Haystead TA, Nakamoto RK, Somlyo AV and Somlyo AP. Acceleration of myosin light chain dephosphorylation and relaxation of smooth muscle by telokin. Synergism with cyclic nucleotide-activated kinase. *J Biol Chem* 273: 11362-11369, 1998.

Wu X, Somlyo AV and Somlyo AP. Cyclic GMP-dependent stimulation reverses G-protein-coupled inhibition of smooth muscle myosin light chain phosphate. *Biochem Biophys Res Commun* 220: 658-663, 1996.

Xu SZ, Zeng F, Boulay G, Grimm C, Harteneck C and Beech DJ. Block of TRPC5 channels by 2-aminoethoxydiphenyl borate: a differential, extracellular and voltage-dependent effect. *Br J Pharmacol*, 2005.

Zhang D, Jin N, Rhoades RA, Yancey KW and Swartz DR. Influence of microtubules on vascular smooth muscle contraction. *J Muscle Res Cell Motil* 21: 293-300, 2000.

Zhang D, Wang Z, Jin N, Li L, Rhoades RA, Yancey KW and Swartz DR. Microtubule disruption modulates the Rho-kinase pathway in vascular smooth muscle. *J Muscle Res Cell Motil* 22: 193-200, 2001.

VITA

Krystina Michelle Berg
10096 Poplar Hall Ct. • Mechanicsville, VA 23116 • 804-221-0579
Fax: 804-828-6455 • kmberg@vcu.edu

Education

PhD Candidate in Biochemistry	August 2002-June 2006
Virginia Commonwealth University	Richmond, VA
Eastern Virginia Medical School	Norfolk, VA
GPA: 3.9	
Bachelor of Science in Biochemistry	May 1997-December 2001
Old Dominion University	Norfolk, VA
Thomas Nelson Community College*	Hampton, VA
St. Petersburg Junior College*	St. Petersburg, FL
* Matriculated Credit to ODU	
GPA: 3.25	

Technical Experience

- *In vitro Confocal and wide-field fluorescence microscopy:*

Fixed tissue samples using a both methanol and paraformaldehyde techniques followed by cryosectioning. Analyzed and interpreted data using confocal, wide-field fluorescent, and DIC microscopy to acquire z-stack images of fixed tissue samples. Performed analysis on 3-D reconstructed volumes using Improvision's software programs Openlab and Volocity. These programs facilitate 3-D reconstruction of both z-series images from confocal microscopy as well as deconvolution of wide-field microscopy.

- *Biochemical Assays*:

Extensive experience in 1-D, 2-D gel electrophoresis, western/immuno blots and various protein staining techniques. Quantification of gels using Bio-Rad's software programs Quantity One and PDQuest for proteomic analysis.

- *In vitro force and intracellular calcium measurements*:

Measured force and Fura-2 calcium signals concurrently in arterial rings or bladder strips during contraction in the presence or absence of attenuating or potentiating pharmacological compounds to elucidate signal transduction pathways.

- *Pressurized artery diameter measurements:*
Travel to Royal Melbourne Institute of Technology, Melbourne, Australia, to learn pressurized artery techniques from Dr. M. Hill. These techniques were then implemented in the lab upon set up of an independent pressurized apparatus. Measured force and diameter simultaneously in artery rings smaller than 200μm.

Research Experience

Biochemistry and Molecular Biophysics Department July 2003-Present
Virginia Commonwealth University Richmond, VA
Graduate Assistant and Ph.D. Student Advisor: Paul H. Ratz, Ph.D.

- Project title: "Mechanism of RhoA kinase-induced length-dependent regulation of tonic force maintenance in Rabbit Epigastric Artery"

Vascular smooth muscle contraction can be divided temporally into early (phasic) and late (tonic) phases. In rabbit epigastric artery, I found the tonic phase to be regulated by stretch. That is, increase stretch of the vessel resulted in a greater tonic force compared to peak force. Using confocal microscopy, 2-D gel electrophoresis and western blot analysis, I found stretch regulates the state of microtubule polymerization and thus determines the activation level of ROK. Increased activation of ROK signal transduction pathways by calcium sensitization mechanisms leads to increased force during the tonic phase of contraction independent of peak force.

Pharmacology Department October 2000-July 2003
Eastern Virginia Medical School Norfolk, VA
Laboratory Technician and Masters Student Advisor: Paul H. Ratz, Ph.D.

- Project title: "2-Aminoethoxydiphenyl Borate (2-APB) Inhibits KCl-Induced Vascular Smooth Muscle (VSM) Contraction"

Sustained tonic force maintenance in vascular smooth muscle in response to K^+ stimulation has long been attributed to L-type voltage operated calcium channels. Using specific pharmacological inhibitors of L-type and transient receptor potential (TRP) channels, I have shown vascular smooth muscle calcium signaling involves both L-type and TRP to regulate sustained calcium entry during a K^+-induced contraction.

Teaching and Leadership Experience

- Co-Instructor undergraduate Biochemistry course (BIOC 403) at Virginia Commonwealth University

- Coordinator for undergraduate biochemistry tutoring. Paired undergraduates with biochemistry graduate students as needed for additional instruction.
- Co-founder of the Biochemistry Graduate Student Council which aims to foster collaborations between students and prepare graduates for successful careers by providing seminars in grant writing and CV preparation.
- Scheduling of apparatus among several graduate students and lab technicians as well as educate new students in both theoretical and practical hands-on aspects of the laboratory.
- Maintained several computers, maintained and developed standardized protocols for microscope based calcium imaging facility, cryostat and myograph apparati.

Professional Membership:

The American Physiological Society 2004-Present
Society for Experimental Biology and Medicine 2006-Present

Awards

American Heart Association July 2005-June 2007
Predoctoral Fellowship ID# 0515275U
Title of Project: "Length-dependant Regulation of
Tonic Force Maintenance in Rabbit Epigastric Artery"

Benjamin W. Zweifach Student Award April 2003
Granted by the Microcirculatory Society San Diego, CA
Awarded for travel to the annual FASEB Conference

Other Experience

Emergency Department June 2002-September 2002
Sentara Norfolk General Hospital Norfolk, VA
Patient Care Technician

- Performed direct venipuncture. Responsible for proper labeling of samples and transportation of blood and other biohazardous materials to the lab for analysis.
- Responsible for introducing intravenous catheters as well as upkeep of IV lines.

Abingdon Volunteer Rescue Squad May 2001-May 2002
Abingdon Volunteer Fire and Rescue Gloucester Point, VA
Emergency Medical Technician-Basic

- Responded to dispatched 911 emergency calls.

- Responsible for direct patient care, bodily substance isolation, and daily inventory of the ambulance.
- Performed general upkeep of the squad house and medic units.
- Certified as an S-2 driver (ambulance)
- State certified Cardiac Tech with over 1300 additional hours of volunteer service.

Publications

Manuscripts submitted or in preparation

K.M. Berg and P.H. Ratz. RhoA Kinase-Induced Length-Dependent Regulation of Tonic Force Maintenance in Rabbit Epigastric Artery Involves Microtubule Depolymerization (*in preparation*)

K.R. Watterson, K.M. Berg, S.G. Payne, S.K. Goparaju, A.S. Miner, P.H. Ratz, S. Milstien and Sarah Spiegel. Sphingosine-1-phosphate and the Immunosuppressant, FTY720 phosphate are Regulators of Detrusor Tone (*in preparation for submission to Journal of Experimental Medicine*)

K.M. Berg, P.F. Blackmore, H.D. White and P.H. Ratz. 2-Aminoethoxydiphenyl Borate (2-APB) Inhibits KCl-Induced Vascular Smooth Muscle (VSM) Contraction (*submitted 08/05 AJP: Heart and Circulatory Physiology*)

M. Porter, M.C. Evans, A.S. Miner, K. M. Berg, K.R. Ward and P.H. Ratz Convergence of Calcium Desensitizing Mechanisms Activated by Forskolin and Phenylephrine Pretreatment, But Not 8-bromo-cGMP. *Am J Physiol Cell Physiol. 2006 Jan 18*

S. Han, K.M. Berg, M. Porter, A.S. Miner, T. Webb and P.H. Ratz. Evidence for Absence of Latchbridge Formation in the Muscular Saphenous Artery. *Am J Physiol Heart Circ Physiol. 2006 Feb 3*

P.H. Ratz, K.M. Berg, N.H. Urban, and A.S. Miner. Regulation of Smooth Muscle Calcium Sensitivity: KCl as a Calcium-Sensitizing Stimulus. *Am J Physiol Cell Physiol 288:C769-C783,2005*

N.H. Urban, K.M. Berg and P.H. Ratz. K^+-depolarization (KCl) Induces RhoA Kinase Translocation to Calveolae and Ca^{2+}-sensitization of Arterial Muscle. *Am J Physiol Cell Physiol 285:C1377-C1385,2003*

Abstracts

K.M. Berg and P.H. Ratz (2006) RhoA Kinase-Induced Length-Dependent Regulation of Tonic Force Maintenance in Rabbit Epigastric Artery Involves Microtubule Depolymerization. *FASEB J* in press

K.M. Berg and P.H. Ratz (2004) Length-dependent Regulation of Force-Maintenance (tonic contraction) in Rabbit Epigastric Artery. *FASEB J 18:699.28*

K.M. Berg and P.H. Ratz (2003) Effects of Y-27632 and GF-109203X in large and small rabbit arterioles. *FASEB J* 17:143a

N.H. Urban, K.M. Berg and P.H. Ratz (2003) KCl-induced ROK Translocation to Caveolae and Calcium Sensitization in Arterial Muscle. *FASEB J* 17:36a

N.H. Urban, K.M. Berg and P.H. Ratz (2003) KCl-Induced Calcium Sensitization via ROK Activation and Translocation. *Biophys J* 84:316a

Poster Presentations

Length-Dependent Regulation of Tonic Force Maintenance in Rabbit Epigastric Artery Involves Microtubule Depolymerization.
October 26, 2005, Watts Day, VCU, Richmond, VA

Length-Dependent Regulation of Force-Maintenance (tonic contraction) in Rabbit Epigastric Artery.
April 19, 2004, Experimental Biology Meeting, Washington D.C.

Effects of Y-27632 and GF-109203X in large and small rabbit arterioles.
April 2, 2003, Experimental Biology Meeting, San Diego, CA

Involvement of RhoA kinase (ROK) and protein kinase C (PKC) in "memory" and myogenic contraction in rabbit Epigastric artery.
October 4, 2002, Research Day, EVMS, Norfolk, VA

Effects of the "Selective Inositol Tris-Phosphate Receptor Inhibitor", 2-APB, on Arterial Contractions Produced by KCl, Phenylephrine, Calcium-depletion and Caffeine.
October 5, 2001, Research Day, EVMS, Norfolk, VA